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(54) Title: MONOCLONAL ANTIBODY TO LYMPHOCYTE-ASSOCIATED CELL SURFACE PROTEIN

(57) Abstract

A hybridoma cell line produced by the fusion of NS-1 myeloma cells with spleen cells obtained from mice immunized with Leukocyte Adhesion Molecule-1 (LAM-1) cDNA transfected cells. The cell line produces a monoclonal antibody reactive with human, monkey, cow, rabbit, sheep, dog, cat, pig and goat LAM-1. The monoclonal antibody produced by the cell line, anti-LAM1-3, may be clinically useful in blocking leukocyte entry into sites of inflammation or tissue injury.

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MONOCLONAL ANTIBODY TO LYMPHOCYTE-ASSOCIATED CELL SURFACE PROTEIN

RELATED APPLICATION

This application is a division and continuation-in-part of U.S. Serial No. 07/313,109 filed February 21, 1989, and entitled LYMPHOCYTE-ASSOCIATED CELL SURFACE PROTEIN.

GOVERNMENT SUPPORT

This invention was made with Government support and the Government has certain rights in this invention under PHS grants CA-34183 and AI-26872.

TECHNICAL FIELD

This invention relates to human lymphocyte associated cell surface proteins. Further, the herein invention relates particularly to a hybridoma cell line and a monoclonal antibody produced by said hybridoma cell line which is useful in detecting immunological disorders symptomatic of disease by identifying T and B cells, for instance, which have reduced LAM-1 expression.

BACKGROUND ART

Genes exclusively expressed by one cell lineage, but not by another, often define the function of that cell population. The generation of genes by the assembly of functionally independent domains has occurred frequently as new genes have evolved to encode proteins with new functions. An inducible endothelial-leukocyte adhesion molecule (ELAM-1) is expressed on the surface of cytokine-treated endothelial cells. This molecule is thought to be responsible for the accumulation of blood leukocytes at sites of inflammation by mediating the adhesion of cells to the vascular lining (Bevilacqua et al., **Proc. Natl. Acad. Sci. USA** 84: 9238 (1987)). A

granule membrane protein found in platelets and endothelial cells, termed GMP-140, has been cloned and is homologous with ELAM-1 (Johnson et al., **Blood Suppl.** 71: 327a (1988)).

DISCLOSURE OF THE INVENTION

The invention generally features a human cDNA sequence encoding lymphocyte-associated cell surface protein LAM-1, which contains domains homologous with binding domains of animal lectins, growth factors, and C3/C4 binding proteins; and the LAM-1 protein encoded by the cDNA sequence or an immunogenic fragment of LAM-1. In a preferred embodiment, the cDNA sequence is isolated from a population of B cell-specific cDNAs from a human tonsil cDNA library, and the amino acid sequence of the protein is subsequently as indicated in Fig.2, more preferably 80% homologous with the sequence shown in Fig. 2 and most preferably 90% homologous. (Here "substantially as indicated" defines a sequence close enough to the indicated sequence to have the same function.)

In another respect, the invention features antibody developed against lymphocyte-associated cell surface protein LAM-1, or a fragment thereof, or against a molecule that specifically associates with LAM-1, or a fragment thereof, to generate a functional molecule.

In another aspect, the invention features a method of identifying cells that express LAM-1 which involves reacting the antibody just described with a population of cells and isolating those that bind the antibody. Binding of antibody can also be used to block the receptor activity of LAM-1.

In another aspect, the invention features a method of treating a human patient suffering from a lymphocyte-mobilizing condition which involves administering a therapeutic amount of an antagonist to LAM-1 in a non-toxic pharmaceutical carrier substance. In preferred embodiments of the method the patient is suffering from

tissue damage, an autoimmune disorder, or cancer, or the patient is an organ or tissue transplant recipient.

In another aspect, the invention features using the cDNA sequence defined above to isolate cross-hybridizing human cDNAs.

In another aspect, the invention features using LAM-1 to identify a ligand which will bind to it or to a molecule that is specifically associated with LAM-1 to generate a functional molecule.

As used herein, the term antagonist includes any agent which interacts with LAM-1 and interferes with its function; e.g., antibody reactive with LAM-1 or any ligand which binds to LAM-1.

Lymphocyte-associated cell surface protein LAM-1 is a unique receptor protein which has not previously been identified. LAM-1 contains domains that are homologous with those found in several different receptors and is a newly described member of a gene family that includes ELAM-1 and GMP-140, proteins which have been implicated in cell adhesion. LAM-1 most likely serves a similar function but is uniquely expressed by lymphocytes. The isolation of cDNA encoding LAM-1 has allowed the determination of the structure of this molecule; the cDNA has been used to transfer expression of LAM-1 to cells that do not express this gene.

Antibodies reactive with LAM-1 can be used to identify cells that express this receptor and to block its function. In addition, the cDNA protein product can be used to develop antagonistic ligands that can interfere with lymphocyte adhesion and function, and thereby be used to treat such conditions as tissue damage and metastasis of cancer cells.

Other features and advantages of the invention will be apparant from the following description of the preferred embodiments thereof and from the claims.

In the drawings, Figs. 1A and 1B show the structure of the LAM-1 cDNA clone.

Fig.2 shows the determined cDNA nucleotide sequence and the deduced amino acid sequence of LAM-1.

Figs, 3A, 3B and 3C show the homologies of LAM-1 and other proteins.

B cell-specific cDNAs were isolated from a human tonsil cDNA library (ATCC No. 37546) using differential hybridization with labelled cDNAs derived from either B cell (RAJI) RNA or T cell (HSB-2) RNA (Tedder et al., **Proc. Natl. Acad. Sci. USA**, 85: 208-212 (1988)). Positive plaques were isolated and cloned, and the cDNA inserts were subcloned into the plasmid pSP65 (Promega, Madison, WI). Nucleotide sequences were determined using the method of Maxam and Gilbert (**Meth. Enzymol.**, 65: 499 (1980)). Gap penalties of -1 were assessed during homology analysis for each neucleotide or amino acid in the sequence where a gap or deletion occurred. One of the 261 RAJI+ HSB2- cDNA clones isolated, B125, contained a 1.90 kb cDNA insert that hybridized with a 2.4 kb RNA species found in several B cell lines (Tedder et al., supra). However, B125 did not hybridize with any of the other RAJI+ HSB2- clones or with mRNA from several T cell lines. The B125 cDNA clone was characterized by restriction mapping and nucleotide sequence determination. A near-full-length 2.3 kb cDNA that hybridized with B125 was isolated, sequenced, and termed pLAM-1.

As shown in Fig. 1A, a restriction map was constructed by the standard single, double or triple digestions of pLAM-1. The putative coding region is shown in black. Arrows indicate the direction and extent of nucleotide sequence determination and the open circles indicate 5'-end labeling. In Fig. 1B, a schematic model of the structure of the LAM-1 mRNA is shown. Thin lines indicate 5' and 3' untranslated sequences (UT), while the thick bar indicates the translated region. The boxes represent the lectin-like and epidermal growth factor (EGF)-like domains and the two short consensus repeat (SCR) units. The open box indicates the putative

transmembrane (TM) region.

The expression of LAM-1 mRNA by cell lines of lymphoid and non-lymphoid origin was examined. Northern Blot analysis revealed that LAM-1 hybridized strongly to a 2.6 kb RNA species and weakly to a 1.7 kb RNA species in poly(A)+ RNA isolated from the B cell lines RAJI, SB, Laz-509 and GK-5. However, RNA isolated from two pre-B cell lines (Nalm-6 and PB-697), three B cell lines (Namalwa, Daudi and BJAB), a myelomonocytic cell line (U937 and U937 cultured with LPS) and an erythroleukemic (K-562) cell line did not hybridize with LAM-1, suggesting that expression of this gene was preferentially associated with B lymphocytes.

The B125 cDNA clone contained a 1,181 bp open reading frame that could encode a protein of 372 amino acids as shown in Fig. 2. The numbers shown above the amino acid sequence designate amino acid residue positions. The numbers to the right indicate nucleotide residue positions. Amino acids are designated by the single-letter code and * indicates the termination codon. The boxed sequences identify possible N-linked glycosylation sites. Hydrophobic regions that may identify signal and transmembrane peptides are underlined. The vertical arrow marks the most probable position of the amino-terminus of the mature protein. (See von Heijne, **Nucleic Acid Res.**, 14: 4683 (1986)).

The amino acid sequence of LAM-1 predicted a structure typical of a membrane glycoprotein. Two potential translation initiation sites were found at nucleotide positions 53 and 92. The second initiation site conformed best to the consensus sequence for optimal initiation (A/G)CCAUG (Kozak, **Cell**: 44" 283-292 (1986)) and was followed by a hydrophobic region of 27 amino acids that may represent a signal peptide. The algorithm of von Heijne predicted that the most probable amino-terminus of the mature protein would be the Trp at amino acid position 52. The LAM-1 sequence contained a second

hydrophobic region between amino acids 346-368 which may be a transmembrane region. The deduced nature LAM-1 protein would have an extracellular region of about 294 amino acids containing 7 potential N-linked carbohydrate attachment sites. LAM-1 would have a cytoplasmic tail of 17 amino acids containing 8 basic and 1 acidic residues. The two cytoplasmic Ser residues may serve as substrates for phosphorylation since protein kinase C phosphorylates Ser residues that are on the carboxyl-terminal side of several basic residues. These results suggest that the processed LAM-1 protein can be isolated by conventional techniques, such as affinity column chromatography with antibody or ligand, from cell lines that normally express this receptor or from transfected cell lines. Or the protein can be synthesized by in vitro translation of the LAM-1 cDNA.

LAM-1 combines previously unrelated domains found in three distinct families of molecules: animal lectins, growth factors, and C3/C4 binding proteins. The proposed extracellular region of LAM-1 contained a high number of Cys residues (7%) with a general structure as diagrammed in Fig. 1B. As indicatead in Fig. 3, segments of homologous proteins are shown with the amino acid residue numbers at each end. Homologous acids are shown in boxes. Gaps (-) have been inserted in the sequences to maximize homologies. The first 157 amino acids of the protein (Fig. 3A) were homologous with the low-affinity receptor for IgE (Kikutani et al. **Cell** 47: 657 (1986)), the asialoglycoprotein receptor (Spiess et al., **Proc. Natl. Acad. Sci. USA** 82: 6465 (1985)) and several other carbohydrate-binding proteins (Drickamer et al., **J. Exp. Med.** 167: 1034 (1988); Krusius et al., **J. Biol. Chem.** 262: 13120-12125 (1987); and Takahashi et al., **J. Biol. Chem.** 260: 12228 (1985)). The amino acids conserved among all animal-lectin carbohydrate recognition domains are indicated (*). Although the sequence homologies were less than 30%, all the invariant residues found in animal

lectin carbohydrate-recognition domains were conserved (Drickamer, **J. Biol. Chem.** 263: 9557 (1988)).

The next domain of 36 amino acids (Fig. 3B) was homologous (36-39%) with epidermal growth factor (EGF) (Gregory, **Nature** 257: 325 (1975)) and the EGF-like repeat units found in Factor IX (Yoshitake et al. **Biochem**, 25: 3736 (1985)) and fibroblast proteoglycan core protein (Krusius et al., supra).

Immediately following these domains were two tandem domains of 62 amino acids each (Fig. 3C) that were homologous with the short consensus repeat units (SCR) that comprise the IL-2 receptor (Leonard et al., **Nature** 311: 626 (1984)), Factor XIII (Ichinose et al., **Biochem.** 25: 4633 (1986)) and many C3/C4 binding proteins (Klickstein et al., **J. Exp. Med.** 165: 1095 (1987), and Morley et al., **EMBO J.** 3: 153 (1984)). In contrast with all of the previously described SCR than contain four conserved Cys residues, these two SCR possessed six Cys residues. The four conserved Cys residues found in all SCR are indicated in Fig. 3C by (*); the additional conserved Cys found in LAM-1 are indicated by (+). Of the multiple SCR present in each of these proteins, the SCR with the highest homology to LAM-1 is diagrammed. A 15 amino acid spacer preceded the putative transmembrane domain.

The deduced amino acid sequence of LAM-1 is homologous with that of ELAM-1 and GMP-140. Thus, these two proteins and LAM-1 define a new family of homologous structures that are expressed by different cell lineages and that can function as receptors in cellular interactions.

Use

As lymphocyte migration and infiltration into areas of tissue damage or injury or tissue transplant can cause or increase pathology, agents that impede these processes can be used as an antigen to produce antibodies against this protein and to develop antagonistic ligands that can

interfere with lymphocyte adhesions and function. The use of these reagents in research will permit the determination of the 3-dimensional structure of LAM-1 and clarify its role in lymphocyte function. The administration of these reagents to patients can be used to block or reduce pathology. As an example, subpopulations of malignant cells that express this antigen would allow the receptor to function in metastasis of tumor cells. Agents developed to block receptor function can inhibit the mestatasis and homing of malignant cells.

CONTINUATION-IN-PART DISCLOSURE

This invention relates to a hybridoma cell line which produces a monoclonal antibody to a human Leukocyte-Adhesion Molecule-1 (LAM-1) [previously called lymphocyte-associated cell surface protein], and to the antibody produced by the cell line. The invention particularly relates to a cell line and a monoclonal antibody produced by the cell line which is useful in the identification and treatment of AIDs by identifying cells, such as, for example, T and B cells, which have reduced LAM-1 expression. Neutrophils can also down-regulate LAM-1 expression following activation. Thus LAM-1 can be a useful marker for identifying neutrophils [6]. For example, the antibody is useful in blocking leukocyte entry into sites of inflammation or tissue damage, and preventing kidney or other organ transplant rejection which is mediated by leukocytes.

Background Art

Applicant's co-pending application Serial No. 07/313,109 describes a human cDNA sequence encoding the lymphocyte-assocated cell surface protein LAM-1, hereafter redesignated the leukocyte-adhesion molecule-1 (LAM-1), which contains domains homologous with binding domains of animal lectins, growth factors, and C3/C4 binding proteins, and the LAM-1 protein encoded by the cDNA sequence. Antagonists to LAM-1 were used in a method of treating a human patient suffering from a

lymphocyte-mobilizing condition which involves administering a therapeutic amount of the antagonist in a non-toxic pharmaceutical carrier.

Normal leukocytes have the ability to leave the circulation and localize in specific lymphoid organs or inflammatory sites through interactions between cell-surface receptors and ligands on endothelial cells [references 1-3, see Glossary]. The Leukocyte-Adhesion Molecule-1 (LAM-1) contains an amino-terminal, lectin-like domain which may interact with specific glyco-conjugates expressed on high endothelial venules (HEV) of peripheral LN (lymph nodes) and activated endothelium [3-5]. LAM-1 is expressed by human peripheral lymphocytes, neutrophils, eosinophils, monocytes and hematopoietic progenitor cells [5-8]. LAM-1 is expressed by the majority of circulating lymphocytes and memory T cells, but is lost following several days of mitogen stimulation [5,9,10]. In contrast, LAM-1 is shed from the cell surface within minutes of exposure of lymphocytes and neutrophils to PMA [5,6,11]. Both lymphocytes and neutrophils express a single LAM-1 protein product, but the molecular weight (Mr) of cell-surface LAM-1 on lymphocytes is 74,000 and that of neutrophils is 90,000-100,000 [6,9,12].

The specific adhesion of some tumor cells to the capillary endothelium and the existence of organ specific metastasis suggest that interactions between tumor cells and normal tissues influence tumor localization [13-15]. Although the molecules that mediate these events in malignant cells have not been completely described, many cell surface molecules involved in the adhesion and migration of normal leukocytes may be involved in the dissemination of hematopoietic malignant cells [1-3]. The mLHR has been implicated in the dissemination of lymphomas [14-16], and a calcium-dependent phosphomannosyl-binding site on human malignant lymphoblastoid cell lines mediates peripheral LN HEV binding

[17]. In the invention described herein, the structure, function and regulation of LAM-1 expression was examined on normal lymphocytes and compared to LAM-1 of malignant leukocytes.

The LAM-1 molecule is a member of a new family of cellular adhesion/homing molecules that contain a lectin-like domain at their amino-terminal end followed by an epidermal growth factor-like domain and short consensus repeat units like those found in C3/C4 binding proteins. In **J. Exp. Med.**, 170: 123-133 (1989) [4] and co-pending Application Serial No. 07/313,109, T.F. Tedder et al. report the isolation and chromosomal localization of cDNAs encoding the novel cell surface molecule LAM-1. In **Eur. J. Immunol.**, 20: 1351-1355 (1990), T.F. Tedder et al. reported that human antigen-specific memory T cells express the LAM-1 necessary for lymphocyte recirculation. In **J. Biological Chemistry**, 265: 7760-7677 (1990), Ord et al. (under the auspices of T.F. Tedder) reported the structure of the gene encoding the LAM-1 of lymphocytes and neutrophils. In **J. Immunology**, 144: 532-542 (1990) [5], T.F. Tedder et al. described two monoclonal antibodies, LAM1-1 and LAM1-2, that react with LAM-1. [Note: As used herein, LAM-1 refers to the leukpocyte-adhesion molecule-1 itself and "LAM1-X" refers to an antibody x which binds to an epitope of LAM-1.}

The monoclonal antibodies LAM1-1 and LAM1-2 were found to be reactive with the majority of blood lymphocytes, NK (Natural Killer) cells, neutrophils, monocytes and hematopoeitic progenitor cells. Binding of LAM-1 may participate in the process of leukocyte extravasation into lymphoid organs or sites of acute inflammation with subsequent loss of LAM-1 from the cell surface. LAM-1 is also recognized by the TQ1 and Leu-8 monoclonal antibodies that have been previously identified.

The loss of LAM-1 expression after leukocyte activation <u>in vivo</u>, with the concomitant increase in

expression of CD2, CD18, CD11a or CD11b may result in significant and dramatic increases in migration and ability to recognize endothelial cell surfaces. Of significance is the fact that patients with AIDs have diminished expression of LAM-1 on their T and B cells. This may also occur in other immunological syndromes. Therefore, alterations in LAM-1 expression by neutrophils are significant because the mLHR is involved in neutrophil migration into sites of acute inflammation. LAM-1, in conjunction with other selectins and receptors, is involved in the extravasation of most leukocytes. The expression of LAM-1 by different leukocytes subpopulations thus plays a key role in determining the characteristics and magnitude of local immune responses [5].

The present invention relates to the production of a new antibody to LAM-1. The new monoclonal antibody, anti-LAM1-3, is useful in radioisotope or immunofluorescent assays for the detection of LAM-1. For example, identifying species which have or do not have LAM-1. The antibody is further useful for separating cells expressing LAM-1 from cells not expressing LAM-1 or visa versa. Furthermore, this monoclonal antibody also completely blocks leukocyte attachment to HEV or endothelium.

Neutrophil-mediated inflammation is involved in a number of human clinical manifestations, including the adult respiratory distress syndrome, multi-organ failure and reperfusion injury. One way of inhibiting this type of inflammatory response would be to block competitively the adhesive interactions between neutrophils and the endothelium adjacent to the inflamed region. Anti-LAM1-3 reacts with LAM-1 on many animal species, but does not bind the mLHR. Anti-LAM1-3 blocks completely lymphocytic traffic to lymph nodes and extravasation of neutrophils from blood to inflammatory sites. The administration of soluble forms of anti-LAM1-3 could be clinically effective for the inhibition of neutrophil-mediated inflam-

mation. Anti-LAM1-3 also blocks lymphocyte adhesion to human HEV and activated endothelium. Therefore, it is likely that the use of anti-LAM1-3 will block lymphocyte entry into sites of inflammation or tissue injury. Such activity will be useful for preventing kidney or other organ transplant rejection which is mediated by lymphocytes.

It is also within the scope of the invention to prepare chimerized monoclonal antibodies from the mouse antibodies. Antibodies are Y-shaped molecules consisting of two long "heavy" chains which define the stem and arms of the Y and two short "light" chains which are attached to the outside of the arms. The amino-terminal ends of the arms of the antibody molecule contain the variable regions of the antibody. The variable regions are specific for a particular antigen. The stem of the molecule is the "constant" region which ends in a carboxylate function (COO^-) and remains the same from molecule to molecule in antibodies of the same isotype in the same species.

The constant region of the mouse antibody has been found to be the primary source human immune reactions to mouse monoclonal antibodies. Using standard genetic engineering techniques, mouse variable regions have been fused to human constant regions to generate "chimeric" (from chimera or chimaera, a monster of Greek mythology which had a lion's head, a goat's body and a serpent's tail) antibodies. These chimeric antibodies thus possess regions of different genetic origin and have been found to have a lower tenency to produce allergic reactions.

Disclosure Of The Invention

A hybridoma cell line produced by the fusion of NS-1 myeloma cells with spleen cells obtained from mice immunized with LAM-1 cDNA-transfected 300.19 cells (a mouse pre-B cell line). The hybridoma cell line produces a monoclonal antibody reactive with human, monkey, cow, rabbit, sheep, dog, cat, pig and goat leukocyte adhesion

molecule-1, LAM-1. The monoclonal antibody produced by the cell line of the claimed invention, identified as anti-LAM1-3, may be clinically useful in blocking leukocute entry into sites of inflammation or tissue injury.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 4 depicts the immunoprecipitation of LAM-1 shed from a cell surface with anti-LAM1 antibodies or a control immunoprecipitation with an unreactive isotype-matched antibody with subsequent sodium dodecyl sulfate-polyacrylamide gel electrophoresis;

Fig. 5 depicts the percentage and reactivity of malignant cells being LAM-1 positive from patients having various forms of hematopoietic malignancies;

Fig. 6 depicts the immunoprecipitation of LAM-1 from the surface of iodinated CLL cells using anti LAM1-1 antibodies or an unreactive isotype-matched control with subsequent sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reduced and non-reduced conditions;

Fig. 7 depicts indirect immunofluorescence results obtained with the anti-LAM1-3 antibody and PPME-FITC; and

Fig. 8 depicts the modulation of cell surface LAM-1 by malignant cells and cDNA transfected cells after PMA exposure.

STATEMENT OF DEPOSIT

A hybrid cell line which produces the anti-LAM-1 monoclonal antibody anti-LAM1-3 embodying this invention was been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 on June 12, 1991 and is assigned A.T.C.C. Deposit No. HB 10771.

BEST MODE FOR CARRYING OUT THE INVENTION

Glossary

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Definitions and Abbreviations

Selectins = a recently described family of cellular adhesion/homing receptor molecules identified by cDNA cloning. Members of this family include the leukocyte adhesion molecule-1 (LAM-1) which is the human homolog of the mouse lymphocyte homing receptor (mLHR), the human granule-membrane protein (GMP-140, PADGEM, CD62) which is expressed on activated platelets and endothelial cells, and the human endothelial leukocyte adhesion molecule-1 (ELAM-1) expressed on activated endothelial cells. The name "selectins" has been suggested for this family because of the presence of the lectin domain and their role in selective cell trafficking.

LN = lymph node

PMA = phorbol 12-myristate 13-acetate

PKC = protein kinase C

LAM-1 = leukocyte adhesion molecule-1

CLL = chronic lymphocytic leukemia

NHL = non-Hodgkin's lymphoma

PPME = poly-phosphomonoester from the yeast HANSENULA cell wall

AML = adult myelogenous leukemia

CML = chronic myelogenous leukemia

PBMC = peripheral blood mononuclear cells

BM = bone marrow.

RPMI Medium = commercial product available from Gibco, Walkersville, Maryland.

FSC = follicular small cleaved cell lymphoma
CSF = colony stimulating factor
DSC = diffuse small cleaved cell lymphoma
FITC = fluorescein isothiocyanate
LPS = lipopolysaccharide
kb = kilobase

Detailed Description

LAM-1 expression was examined on normal and neoplastic leukocytes to further understand the mechanisms that regulate leukocyte migration. The immunoprecipitation of a large fragment of LAM-1 of molecular weight 69,000 from the supernatant liquid of normal lymphocytes cultured with PMA demonstrated that LAM-1 can rapidly be cleaved from the cell surface (Fig. 4). That the LAM-1 expression is down-modulated by shedding rather than by internalization suggests that a PMA-sensitive regulatory pathway which is distinct from that which regulates down-modulation of most other surface molecules, controls the expression of LAM-1. This regulatory pathway may specifically involve the activation of PKC (Table 3). The presence of the soluble isoform of LAM-1 in the supernatant fluid of lymphocytes cultured without stimulation (Fig. 4) suggests that LAM-1 may also be continuously shed at a slow rate with its expression kept constant by the continuous synthesis of new receptors. Although the mechanism of shedding is unknown, enzymatic cleavage of the cell-surface receptor may result from the specific activation of a membrane bound protease. This is a likely method since a soluble protease secreted by activated leukocytes was not detected in this work. Alternatively, activation-induced changes in the comformation of the LAM-1 protein may expose nascent sites on LAM-1 that are then susceptible to cleavage by soluble proteases. Nonetheless, the finding that cell lines transfected with LAM-1 cDNAs rapidly modulate LAM-1 expression after PMA exposure (Fig. 8) suggests that the

protease which cleaves LAM-1 is ubiquitous in distribution. The down-modulation of LAM-1 by shedding is similar to that of mLHR [33-34]. This is consistant with prior observations that the cell surface expression of LAM-1 is rapidly down-regulated upon activation [5,6,11]. LAM-1 removal from the cell surface may thus be necessary for the detachment of leukocytes from the endothelium so as to allow for their subsequent migration into tissues.

LAM-1 was most frequently expressed by CLL cells among the various hematologic malignancies studied (Table 1, Fig. 5). These results extend previous studies of LAM-1 expression to TQ1 and Leu-8 using CLL and NHL cells [35-37]. Since the expression of LAM-1 was somewhat restricted among hematologic malignancies, the expression, or absence of expression, may have a major impact on the trafficking of leukemic cells and the dissemination of NHL. Immunoprecipitation of LAM-1 from CLL cells showed that it resembled the Mr 74,000 isoform of the glycoprotein expressed by normal lymphocytes (Fig. 6). In addition, LAM-1 expressed by malignant cells was functional since LAM-1 on normal lymphocytes and CLL cells were both able to bind HEV and PPME (Table 2, Fig. 7). Both HEV and PPME binding was mediated by LAM-1 since the new monoclonal antibody, anti-LAM1-3, was able to completely block all HEV and PPME binding.

Table 1. LAM-1 Shedding Is Blocked By Protein Kinase Inhibitors

	Without PMA Treatment[a]		With PMA Treatment	
Cells with	% Positive	MCF No.	% Positive	MCF No.
Medium	60	115	13	47
Staurosporine 1mM	57	91	54	95
500μM	72	95	49	91
100μM	51	88	51	92
50μM	47	92	58	100
H-7 1mM	52	94	53	92
500μM	67	102	32	80
100μM	58	109	23	91
50μM	61	106	11	61
NaN_3 1μM	59	113	20	50

a = The percentage of cells reactive with the anti-LAM1-1 monoclonal antibody was determined by indirect immunofluorescence analysis. The relative intensity of staining of the positive cells is indicated based on the mean fluorescence channel number (MFC No.) obtained with FACS analysis (256 channels, on a 3-decade log scale. Cells treated with an unreactive monoclonal antibody had 3% positive cells with a MFC no. of 40.

Table 2. Binding Of Normal And Malignant Cells To HEV.

	LAM-1 Expression[a]		CD44 Expression		Cells Bound per HEV
Test	% Pos.	Intensity	% Pos.	Intensity	
1	70	+++	93	++++	2.12 ± 0.05
2	59	++	99	++++	1.02 ± 0.22
4	52	++	96	++++	1.02 ± 0.32
3	2	-	65	++++	0.18 ± 0.11
Normal PBL	85	+++	100	++++	4.64 ± 0.67

a = The percentage of cells with the anti-LAM1-1 and 515 monoclonal antibodies were determined by indirect immunofluorescence analysis. The relative intensity of staining of the positive cells is indicated based on a (-) being no reactivity and (++++) indicating the highest reactivity.

b = Values represent the mean number of cells (± SD) bound to each HEV. A total of 150 HEV were examined in each sample. The difference between the number of cells bound per HEV in LAM-1+ cases (1,2 and 4) and the LAM-1- case (3) was statistically significant ($P < 0.005$) using Student's t-test statistic.

TABLE 3

EXPRESSION OF ADHESION MOLECULES BY MALIGNANT LEUKOCYTES

	No of cases expressing antigen / number examined[a] (mean % among positive cases)						
Diagnosis	LAM-1	CD44	CD11a	CD11b	CD18	CD54	CD58
Pre-B-ALL							
CD10+	4/15 (32)	10/12 (67)	1/8 (46)	0/15	1/12 (40)	4/13 (33)	6/13 (69)
CD10-	0/6	5/5 (80)	0/6	0/5	0/5	0/5	2/5 (55)
T-ALL	2/10 (31)	8/9 (67)	3/8 (59)	0/2	5/10 (49)	4/10 (46)	1/10 (62)
B-CLL	16/27 (55)	16/18 (76)	3/14 (45)	0/21	3/18 (32)	1/18 (31)	1/18 (43)
B-lymphoma							
FSC	6/12 (32)	9/11 (59)	8/9 (56)	0/12	9/11 (38)	5/11 (50)	2/11 (49)
DSC	2/4 (41)	4/4 (70)	1/4 (90)	0/3	1/4 (90)	3/4 (40)	1/4 (77)
DLC	1/6 (27)	6/6 (59)	5/6 (55)	0/6	4/4 (49)	3/6 (54)	2/6 (53)
Burkitt's	0/4	2/4 (52)	0/4	0/4	0/4	1/4 (72)	0/4
M. Myeloma	0/3	2/2 (84)	3/3 (53)		2/2 (37)	0/3	1/3 (52)
AML	2/19 (44)	16/16 (79)	6/11 (50)	5/19 (42)	11/16 (41)	1/16 (47)	9/16 (61)
CML	0/12	10/10 (61)	5/10 (43)	2/12 (48)	5/10 (39)	0/10	4/10 (70)

[a]Cases were considered positive for the Ag being examined if > 25% of the cells were positive.

Table 4. PPME and HEV binding are enhanced by lymphocyte aCtivation.[1]

	Binding of [^{125}I]PPME (c.p.m. ± s.d.)			Cells Bound/HEV[2] (± s.d.)	
Stimulus	Medium	anti-LAM1-3	EDTA	Medium	anti-LAM1-3
Medium	4847±372	1160±104	578	1.4±0.2	0.2±0.2
anti-LAM1-10	6868±571	2103±905	2030	1.5±0.8	0.2±0.2
anti-CD2	17886±419	2863±689	965	2.4±0.9[4]	0.2±0.2
anti-CD3	19718±1294	1211±618	303	2.7±1.0[3]	0,2±0.2

1. Blood lymphocytes were isolated, incubated for 20 minutes at 4°C with the indicated antibodies, and anti-CD3 was crosslinked as shown by Spertini et al., Nature 349: 691-694 (1991) [See Spertini et al., Fig. 1]. After one wash, the cells were incubated with ^{125}I-labelled PPME 90.36 μg/ml, 2.2x10^{5} c.p.m. per sample) at 4°C for 30 minutes. Anti-LAM1-3 was added 1 minute before the addition of the test antibody and during all incubations. The calcium-independent binding of [^{125}I]PPME was assessed in the presence of 5 mM EDTA. Cells were washed, resuspended in PBS-BSA and layered on a 750-μl cushion of 75% (v/v) calf serum. The cell pellet was isolated and bound [^{125}I]PPME assessed by γ (gamma) counting. These data are representative of those obtained in three experiments. Fluoroscein-labelled PPME was iodinated by standard methods, the specific activity (2x10^{4} c.p.m./ng) determined by self-displacement curve analysis, and the maximum binding capacity was 20%.

2. HEV binding was assessed using 12-μm freshly cut, frozen rat lymph node sections. The number of lymphocytes bound to HEV was counted on coded

slides. Values are means ± standard deviation (s.d.) of four experiments, and the differences between control antibody-treated cells and anti-CD2 and anti-CD3 treated cells were significant.

3. $P<0.05$ using the paired Student's t-test.

4. $P<0.01$ using the paired Student's t-test

The level of HEV binding was also proportional to the quantity of LAM-1 expressed, and LAM-1 negative cells were unable to bind HEV and PPME. LAM-1 was also shed from the surface of CLL cells following PMA exposure (Fig. 8). However, the signalling pathway for shedding may be less active in some CLL cells since the time-course of LAM-1 shedding was slower than in normal lymphocytes. Malignant cells, therefore, express functional LAM-1 receptors that are indistinguishable from their normal counterparts on normal cells and the expression of LAM-1 by CLL cells correlated with the high tendency of these cells to localize into peripheral LN.

In contrast to LAM-1, CD44 expression was found to be consistently expressd at high levels among the leukemias and NHL examined, while the expression of other adhesion molecules CD11/CD18, CD54 and CD58 was variable (Table 1). Expression of CD44 did not correlate with the ability of cells to bind to HEV since LAM-1 negative CLL cells that expressed high levels of CD44 did not bind to HEV in frozen section assays (Table 2) similar to what was shown by one research group using lymphoblastoid cell lines [17]. CD44 constitutes a broadly distributed family of glycoproteins expressed on virtually all hematopoietic cells, fibroblasts, epidermal, glial and melanocytic origin cells [21,38]. Although CD44 was initially regarded as the human homing receptor equivalent of the mLHR [28,29], it may be more generally involved in cell-cell or cell-matrix binding as a receptor for hyaluronate [39]. Previous studies have also suggested that CD44 is involved in the dissemination of NHL [40]. During the work resulting in the present in-

vention, however, no clear relationship could be inferred from the results of CD44 expression alone.

LAM-1 is expressed on most neutrophils, monocytes, normal myeloid progenitor cells and early erythroid precursors in BM (bone marrow) [6]. The co-expression of this homing receptor and other adhesion molecules may control the physiological retention (homing) of these cells in BM. The homing of intravenously transplanted hematopoietic stem cells is mediated by a recognition system with galactosyl and mannosyl specificities [41] which might also mimic the LAM-1 ligand [42]. In this regard, it is noted that AML and CML cells were found to lack expression of LAM-1. Unlike the situation with lymphoid tumors, this is in sharp distinction with the high level expression of LAM-1 on normal myeloid cells. The absence of LAM-1 expression on most AML and CML cells might favor the passage of these cells into the bloodstream. Although overnight culture of CML cells did not result in the expression of LAM-1 on the cell surface, the overall lack of LAM-1 expression by these cells indicates that further investigations of the regulation of LAM-1 by leukemic myeloid cells is warranted.

There is growing evidence in mouse and man that the binding of lymphocytes to HEV of peripheral LN and the migration of normal leukocytes from blood into inflammatory lesions is controlled by several adhesion molecules whose expression is coordinately regulated [5,6,33,43]. It is likely that similar mechanisms will contribute to the spread of leukemias and lymphomas. In one murine study, the expression of functional receptors for HEV was shown to control the hematogenous dissemination of malignant lymphocyte populations to HEV bearing organs [16]. Lymphomas that bound well to HEV disseminated weakly vias the blood, ultimately involving all LN groups symmetrically. In contrast, gross involvement of LN by non-binding lymphomas was limited to nodes draining localized tumors which formed at the site of in-

jection. These results suggested that the expression of functional receptors for HEV either controls the hematogenous dissemination of malignant lymphocyte populations to HEV-bearing organs, or is co-regulated with factors that determine metastatic behavior [16]. In humans, the expression of functional HEV binding molecules such as LAM-1, on CLL and low-grade lymphoma cells may also contribute to the wide-spread dissemination of these malignant cells to LN as occurs with normal lymphocytes.

Materials and methods

Cell Samples

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation of blood, bone marrow (BM) samples and single cell suspensions of LN. Cells were obtained by protocols approved by the Human Protection Committee of the Dana-Farber Cancer Institute. Tumor type was classified according to conventional morphological, cytological and immunophenotype criteria. Tumor cell lineage was determined by analysis of antigens (Ag) including surface and cytoplasmic immunoglobulin (Ig), HLA-DR Ag, CD1, CD2, CD3, CD4, CD5, CD6, CD8, CD9, CD10, CD11b, CD13, CD14, CD19, CD20 and CD33. Cells were examined immediately after isolation or were immediately cryopreserved and kept frozen in liquid nitrogen until used. The frequency of malignant cells was always greater than 90% in every sample examined.

Antibodies.

The anti-LAM-1 monoclonal antibodies anti-LAM1-1 and anti-LAM1-2 and the monoclonal antibody anti-TQ1 have been previously described [5,8]. The anti-LAM1-3 antibody (IgG1) of the claimed invention was generated by the fusion of NS-1 myeloma cells with spleen cells from Balb/c mice that were repeatedly immunized with cells of the mouse pre-B cell line 300.19 transfected with a LAM-1 cDNA as described [5]. The antibodies used in these

studies included: 2F12 (CD11a) and 10F12 (CD18) [18] which were gifts from J. Ritz (Dana-Farber Cancer Inst., Boston MA); TS2/9 (CD58, anti-LFA-3) [19] and RR 1/1 (CD54, anti-ICAM-1) [20] which were gifts from T.A. Springer (Center for Blood Research, Boston, MA); 515 (CD44) [21] a gift from G.S. Kansas (Dana-Farber Cancer Inst.); and 904 (CD11b) [22].

Immunofluorescence analysis.

Indirect immunofluorescence analysis was performed on viable cells isolated by Ficoll-Hypaque density gradient centrifugation. The expression of LAM-1, CD11a, CD11b, the β subunit of CD11 complex (CD18), CD44, CD54, and CD58 was examined by indirect immunofluorescence with flow cytometry analysis (Coulter Epics C, Coulter Electronics, Hialeah FL). Isotype-matched murine antibodies that were unreactive with human leukocytes were used as negative controls. Cells were incubated with each monoclonal antibody for 20 minutes on ice, washed, and treated with FITC-conjugated goat anti-mouse Ig reagents (Southern Biotechnology Associates, Birmingham, AL).

The ability of normal lymphocytes and B-CLL cells to bind the fluorescein derivative of PPME (fl-PPME) (a gift of S.D. Rosen, University of California, San Francisco, CA) was assessed by incubating cells for 30 minutes on ice with 100 μl of fl-PPME at 30 μg/ml in phosphate buffered saline (PBS). After washing twice, the binding of fl-PPME was examined by flow cytometry analysis as described [23,24].

HEV binding assay.

The in vitro HEV binding assay was performed using frozen tissue sections of human or rat peripheral LN using the methods of Stamper and Woodruff [25] and Butcher, et al. [26] as described [5]. Blocking of cell binding using the anti-LAM-1 monoclonal antibodies was carried out using freshly cut-frozen rat lymph node sec-

tions and the antibodies were used as ascites fluid at dilutions of 1:100.

Immunoprecipitation analysis

Cells were washed twice, resuspended in RPMI 1640 medium (Sigma, St. Louis, MO) at a concentration of 30×10^6 cells/ml) and treated for 40 minutes at room temperature with neuraminidase (0.1 U/ml, Calbiochem, La Jolla, CA) and then labelled by lactoperoxidase-catalyzed iodination. After washing, the cells were lysed in buffer containing 1% (v/v) NP-40 as described [27]. Cell lysates were precleared for 2 hours using 3 µl of murine ascites fluid (isotype matched antibody) and 25 µl of a 50% (v/v) suspension of Gammabind-G Agarose (Genex, Gaithersburg, MD). Cell lysates were precleared again overnight. Half of the precleared lysate was then incubated with 3 µl of anti-TQ1 ascites fluid, 3 µl of anti-LAM1-1 ascites fluid, and 50 µl of Gammabind-G with constant rotation at 4°C for 18 hours. The other half of the lysate was treated similarly using 3 µl of isotype-matched murine ascites fluid. Immunoprecipitates were washed and analyzed by SDS-PAGE. Molecular weights (Mr) were determined using standard molecular weight markers (BRL, Bethesda, MD).

In experiments designed to study LAM-1 shedding, LAM-1 was immunoprecipitated as described above from the supernatant fluid and the pellet of PBMC that had been cultured for 60 minutes at 37°C in RPMI 1640 medium alone or in RPMI medium containing PMA (100 ng/ml, Sigma, St. Louis, MO). In addition, expression of LAM-1 was assessed after incubation of the cells with PMA (10 nM for 30 minutes) following the prior culture of the cells with sodium azide (Sigma) or the protein kinase inhibitors, 1-(5-Isoquinolinyl-sulfonyl)-2-methylpiperazine (H-7, Calbiochem) and staurosporine (Sigma) for 30 minutes at 37°C.

Discussion of Figures 4-8.

Figure 4.

LAM-1 is shed from the cell surface into the culture medium. PBMC were surface iodinated and cultured for 60 minutes in medium or medium containing 100 ng/ml PMA. The supernatant fluid and cells were harvested and immunoprecipitated with anti-LAM-1 antibodies or an unreactive isotype matched control antibody (Cont.). Immunoprecipitated materials were electrophoresed on a 7.5% SDS acrylamide gel under reducing conditions followed by autoradiography. The migration of known molecular weight standards are shown in kilo-Daltons (kDa).

Figure 5.

The frequency of LAM-1 expression by malignant cells. Cells from 118 patients with various forms of hematopoietic malignancies were examined for surface LAM-1 expression using the anti-LAM1-1 monoclonal antibody in indirect immunofluorescence assays with flow cytometry analysis. In each instance, the background staining for each sample was determined using an unreactive isotype-matched monoclonal antibody and the level of background staining (usually less than 5%) was subtracted from the values shown. The horizontal bars represent the mean frequency of reactive cells. [Abbreviations: Pre-B, pre-B acute lymphoblastic leukemia; T-ALL, T cell acute lymphoblastic leukemia; B-CLL, B type chronic lymphocytic leukemia; FSC, follicular small cleaved cell lymphoma; DSC, diffuse small cleaved cell lymphoma; DLC, diffuse large cell lymphoma; Burk., Burkitt's type lymphoma; M.M., multiple myeloma; AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia]. The relative fluorescence staining intensity of the malignant cells is indicated where the positive population could be identified as a distinguishable peak from background fluorescence staining: ±, where a shoulder of positively stained cells was evident, +, where a separate peak of positive

cells was identified with weak fluorescence; ++, a definite separate peak of fluorescence positive cells of moderate fluorescence; +++, a peak of fluorescence positive cells of the same intensity as normal blood lymphocytes. The tissue source of all malignant cells is also indicated.

Figure 6.

Anaylsis of LAM-1 immunoprecipitated from B-CLL cells. Detergent lysates of surface iodinated cells (45 x 10^6) were immunoprecipitated with the anti-TQ1 and anti- LAM1-1 antibodies (LAM-1) or an unreactive isotype-matched antibody control (Cont.). Immunoprecipitated materials were divided and analyzed under non-reducing and reducing conditions on a 12% SDS polyacrylamide gel followed by autoradiography. Molecular weights (kDa) were determined by the migration of known protein standards.

Figure 7.

Normal human lymphocytes and CLL cells are capable of binding PPME through LAM-1. Cells were examined for LAM-1 expression by indirect immunofluorescence analysis after treatment with the anti-LAM1-3 monoclonal antibody (dark line) or with an unreactive isotype matched antibody (thin line). Cells were also reacted with FITC-conjugated PPME after treatment with the anti-LAM1-3 antibody (thin line) or an unreactive control antibody (dark line). The fluorescence intensity of cell staining was analyzed by flow cytometry.

Figure 8.

Modulation of cell surface LAM-1 by malignant cells and cDNA transfected cells after PMA exposure. Cells transfected with LAM-1 cDNA, K562-LAM1 and 300.19-LAM1, and malignant cells that expressed LAM-1 were either cultured for 90 minutes in media or in media containing 10 ng/ml PMA. Following culture, the cells were examined for LAM-1 expression using the anti-LAM1-1 antibody in indirect immunofluorescence assays with flow

cytometry analysis. Cells were also stained with an unreactive control antibody and the level of background staining was always less than 5%. The frequency of cells expressing LAM-1 is shown with the number of background staining cells subtracted.

Results

LAM-1 is released from the cell surface following PMA exposure.

Immunoprecipitation experiments were carried out to determine the fate of LAM-1 after modulation from the surface of PBMC exposed to PMA [5]. Surface iodinated cells were cultured for 60 minutes in RPMI medium alone or medium containing PMA. After culture, the supernatant fluid and cells were separated, and the cells were lysed with detergent. The cell lysate and supernatant fluid were immunoprecipitated with a combination of anti-LAM1-1 and anti-TQ1 antibodies that bind to different epitopes of LAM-1 [5], and together are more efficient for immunoprecipitation. The cells were also treated with neuraminidase prior to surface iodination since LAM-1 may be more readily immunoprecipitated after the removal of sialic acid residues. Treatment of cells with PMA resulted in a dramatic loss of immunoprecipitable LAM-1 from the cell surface with a concomitant increase in the level of LAM-1 precipitated from the supernatant fluid. Incubation of cells in medium without PMA also resulted in a small amount of LAM-1 being found in the supernatant fluid (Fig. 4). The molecular weight of LAM-1 precipitated from the supernatant fluid was slightly smaller (by about 5 kDa) than the species of LAM-1 found on the cell surface. Interestingly, the residual LAM-1 found on the cell surface of PMA-treated cells was most similar in molecular weight to that of the LAM-1 found in the supernatant fluid. The quantititive recovery of labeled LAM-1 from the supernatant fluid, in comparison to the amount immunoprecipitated from

solubilized cells, demonstrates that a major portion of LAM-1 is shed from the cell surface and not internalized following PMA exposure.

Expression of adhesion molecules by malignant leukocytes.

The expression of LAM-1 and other cell surface molecules known to be involved in lymphocyte adhesion and migration was examined on malignant leukocytes from 118 patients by indirect immunofluorescence analysis. LAM-1 expression was most frequently demonstrated on CLL cells and among lymphomas classified as follicular (FSC) and diffuse small cleaved cell lymphoma (DSC) (Table 3). On the other hand, most acute myeloblastic leukemia (AML), acute lymphoblastic leukemia (ALL), chronic myelocytic leukemia (CML), diffuse large cell lymphomas (DLC), Burkitt's lymphomas and multiple myelomas were LAM-1 negative. The level of cell surface LAM-1 expression was highest on CLL cells; and in 11 of 16 cases that expressed LAM-1, more than 50% of cells were LAM-1+ (FIG. 5). In general, the fluorescence intensity level of LAM-1 staining correlated with the frequency of LAM-1+ cells such that most malignant cell populations with less than 25% positive cells failed to express LAM-1 at easily detectable levels.

CLL cells rarely expressed adhesion molecules other than LAM-1, with the exception of CD44 (Table 3), which has also been associated with lymphocyte homing [28,29]. More than 90% of the cell samples were CD44+, consistent with its ubiquitous distribution on normal hematopoietic cells. Ninety-three percent of the leukemias and 84% of the B-NHL were positive for this antigen and generally greater than 60% of the cells were CD44+. In general, the expression of CD11a, CD11b, CD18, CD54, and CD58, was more heterogeneous (Table 3).

Incubating normal blood lymphocytes for about 8-16 hours at 4°C can result in complete loss of LAM-1 from

the cell surface [30]. However, the cryopreservation of PBMC, LAM-1+ lymphoblastoid cell lines and freshly isolated LAM-1+ malignant cells did not appreciably alter the qualitative expression of LAM-1. A minor quantitative decrease in LAM-1 expression was observed that could be reversed by culturing the cells for about 8-16 hours in RPMI 1640 medium with 10% FCS (fetal calf serum). Consequently, 37 of the samples of malignant cells were re-examined following the above culture. This treatment, however, did not result in the appearance of LAM-1 on the cell surfaces or a significant increase in the frequency of LAM-1 expression in any case.

Structure of LAM-1 expressed by malignant cells.

Anti-LAM-1 antibodies were used to immunoprecipitate LAM-1 from CLL cells. LAM-1 migrated with a Mr of 68,000 under non-reducing conditions and at 73,000 after reduction (FIG. 6), similar to LAM-1 immunoprecipitated from normal lymphocytes (FIG. 4). Therefore, it appears that normal and malignant lymphocytes express the same cell-surface LAM-1 protein.

LAM-1 receptor function.

The relationship between LAM-1 expression and the ability of cells to bind to human peripheral LN HEV was examined using cells from normal circulating blood, three LAM-1 positive CLLs and one LAM-1 negative CLL. Cells were assessed for their ability to bind HEV of human peripheral LN using the frozen section assay of Stamper and Woodruff [25]. The LAM-1+ cells bound to HEV at levels which corresponded to the amount of LAM-1 expressed on their cell surface, while the LAM-1 CLL cells did not bind (Table 2). In contrast, CD44 expression was quite high on all of the cell samples examined and did not correlate with HEV adhesion. Additional studies examined the ability of anti-LAM-1 monoclonal antibody to block HEV binding. A new antibody, anti-

LAM1-3, was able to specifically block 92 to 95% of normal lymphocyte and LAM-1+ CLL cell binding (cells from Table 2) to rat peripheral LN HEV. In contrast, the binding of a different antibody, anti-LAM1-10, reactive with a different epitope of LAM-1, had no detectable effect on HEV binding. (See Table 4). Therefore, the levels of LAM-1 expression correlated with the ability of cells to bind HEV and antibodies reactive with LAM-1 specifically blocked binding.

The ligand for the mLHR is mimicked by the mannose 6 phosphate-rich polysaccharide PPME [24]. Therefore, the ability of normal human lymphocytes and CLL cells to bind fluoresceinated PPME was examined to further characterize the functional capacity of human LAM-1. Both normal blood lymphocytes and LAM-1+ CLL cells were able to bind PPME, while LAM-1 CLL cells did not bind PPME (FIG. 7). The specificity of PPME binding to LAM-1 was verified by the ability of anti-LAM1-3 antibody to completely block PPME binding to the cells (FIG. 7).

Modulation of LAM-1 expresion.

The exposure of normal lymphocytes and neutrophils to phorbol esters induces a rapid disappearance of LAM-1 from the cell surface [5.6]. Therefore, modulation of LAM-1 expression after PMA stimulation was investigated on cells from 16 patients with CLL. After 90 minutes exposure to PMA, LAM-1 expression was completely lost in 4 cases, whereas it was only partially down-modulated in the 12 remaining cases. In 10 of these 12 cases, the down-modulation of LAM-1 expression was similar to that of RAJI cells cultured simultaneously under the same conditions (FIG. 8), while it was only minimal in two cases (B-CLL #1 and #2, Fig. 8). In cells from one patient with a FSC type NHL, PMA induced an almost complete modulation of LAM-1 expression after 90 minutes of stimulation (Fig. 8). In six LAM-1+ CLL cell samples tested further, PMA exposure lead to the complete loss of

LAM-1 expression after 180 to 360 minutes of culture with PMA with similar kinetics to those of RAJI cells treated simultaneously. These experiments were also carried out using cryo-preserved blood lymphocytes and RAJI cells as controls, with no appreciable affect on the ability of the cells to modulate LAM-1 expression after PMA exposure.

The down-modulation of LAM-1 expression was also investigated in LAM-1 cDNA transfected cells. The erythroleukemia cell line, K562, and the mouse pre-B cell line, 300.19, were transfected with LAM-1 cDNA as described [5], generating cells that express relatively high levels of cell surface LAM-1 (Fig. 8). In contrast to what was observed with RAJI cells and the majority of CLL cells, 90 minutes exposure of these cells to PMA induced an almost complete loss of LAM-1 from the cell surface.

The role of PKC in LAM-1 shedding was further assessed by culturing normal blood lymphocytes with protein kinase prior to their exposure to PMA. Treatment of cells with both H-7 [31] and staurosporine [32] inhibited shedding, albeit at different optimal molar concentrations (Table 1). In contrast, pretreatment of lymphocytes with sodium azide did not inhibit down-modulation of cell surface receptor. However, the shedding process required on-going metabolism since PMA treatment at 4°C did not induce detectable LAM-1 shedding (data not shown). Thus, PKC may regulate cell surface receptor expression through direct phosphorylation of LAM-1 which may signal for cleavage or through kinase reguation of protease activity.

Shedding of LAM-1 does not result from the activation-induced secretion of a soluble protease. Neutrophils (10^7/ml) were activated with lineage-specific cytokines such as granulocyte/macrophage-CSF, to indue complete LAM-1 shedding [6]. The supernatant fluid of these cultures was harvested and used as culture medium for lymphocytes or LAM-1 cDNA transfected cells for 120

minutes at 37°C. This treatment did not induce detectable LAM-1 shedding from the surface of lymphocytes as assessed by flow cytometry analysis. In addition, the activation of neutrophils by lineage-specific stimuli in the presence of lymphocytes failed to induce detectable loss of lymphocyte LAM-1 while neutrophil shedding of LAM-1 was complete. Thus, it appears that a membrane anchored protease cleaves LAM-1 from the cell surface or that cellular activation is required for cleavage to occur.

LAM-1 expression on malignant leukocytes from tissue.

LAM-1 expression may be down-regulated during lymphocyte entry into tissues and this down-regulation is reversible in culture [5]. Malignant cells isolated from the highly infiltrated spleen of a CLL patient were found to express LAM-1 at a lower level than the CLL cells found in his peripheral blood (i.e 65% on blood cells and 25% on splenocytes). The percentage of LAM-1 positive spleen cells was comparable to that of the patients peripheral B-CLL cells stimulated for 180 minutes with PMA. This suggests that LAM-1 expression was decreased with entry of the CLL cells into the spleen as occurs with normal lymphocytes.

LN cells from patients with NHL (two patients with FSC, one with DLC and two with DSC) and leukemia BM cells (from two patients with AML, one with ALL and one with CML) were incubated overnight at 37°C in RPMI 1640 medium containing 10% FCS. Contrary to the results obtained under the same conditions with normal spleen cells [5], an increase in LAM-1 expression was not detected on the malignant cells after culture, suggesting that LAM-1 was not expressed constitutively on these cells.

Table 5, below, lists a number of the properties of the monoclonal antibodies anti-LAM1-1, -2 and -3. Lines 1, 2, and 3 give the name of the antibody, the isotype

and the differences in staining intensity. These properties are not necesarily indicative of differences in epitope recognition.

Functional studies of the antibodies are given in lines 4-6. These results demonstrate that different parts of the LAM-1 molecule are recognized by the different monoclonal antibodies. Lymph nodes contain structures called high endothelial venules (HEV) which are utilized by lymphocytes to enter the lymph nodes (the site of immune responses) from the blood stream. Emigration of lymphocytes into the node has been shown to be mediated by adhesion molecules which allow the cells to stick to and then traverse the venule. This process has been studied by incubating isolated lymphocytes with lymph node tissue sections. When the sections are incubated with lymphocytes alone, the cells will adhere to HEV, and the number of adherent cells can be counted. Various monoclonal antibodies, including the LAM-1 antibodies, have been used to block this binding, Line 4 gives the results of such studies for LAM1-1, -2 and -3.

The polysaccharide PPME mimics the natural ligand for the LAM-1 molecule. Since PPME can be directly fluoresceinated, it is possible to study the effect of the various monoclonal antibodies on the interaction of LAM-1 and PPME. Line 5 details the results using cells which were first incubated with a LAM-1 monoclonal antibody, followed by treatment with PPME-FITC. Anti-TQ1, anti-LAM1-2 and anti-LAM1-3 blocked PPME binding and anti-LAM1-1 enhanced PPME binding. These results demonstrate the functional (and by extrapolation, the specificity) differences between the antibodies.

The results shown on line 6 were obtained by the reverse of the line 5 experiment; i.e., cells were first incubated with unlabelled PPME, followed by indirect immunofluorescence with the LAM-1 antibodies. As in line 5, differences in the effects of incubation with PPME on subsequent monoclonal antibody binding indicates that the

various monoclonal antibodies recognize different epitopes of the LAM-1 molecule.

Lines 7-10 detail the results of studies in which the ability of a given monoclonal antibody to block the subsequent binding of other monoclonal antibodies was analyzed. Blocking of one antibody by another provides evidence that the two antibodies in question recognize epitopes which are identical or close together on the molecule. The results in Table 4 indicate that anti-LAM1-1 does not block anti-LAM1-3, indicating that their epitopes are different. Anti-LAM1-2, however, does block anti-LAM1-3, indicating that these epitopes are at least close to each other. There are differences between anti-LAM1-2 and anti-LAM1-3, however, because of the difference response they generate regarding Leu 8. Anti-LAM1-2 does not at all block Leu 8, whereas anti-LAM1-3 strongly blocks it. Species cross-reactivity gives further indications of the diferences which exist between the antibodies and the epitopes that they identify.

Line 11 gives the results of the domain mapping regarding the monoclonal antibodies. The LAM-1 molecule contains three domains which are:

(a) a lectin-like domain (L);

(b) an epidermal growth factor-like domain (EGF); and

(c) a domain of short consesus repeats (SCR).

In order to determine which domain was recognized by each antibody, cDNAs were constructed which contained the information coding for:

(1) the whole LAM-1 molecule;

(2) the L, EGF and SCR domains from the LAM-1 molecule;

(3) the L domain from the LAM-1 plus EGF and SCR domains from the CD62 molecule of the same family of proteins;

(4) the L plus EGF domains from LAM-1 (SCR from CD62; and

(5) the L plus SCR domains from LAM-1 (EGF from CD62).

These cDNAs were transfected into cells which then produced the corresponding proteins. The pattern of reactivity of the various monoclonal antibodies was then determined as shown in Table 6, and the domain necessary for monoclonal antibody reactivity was assigned. For example, anti-LAM1-3 bound to cells expressing all the domains described with medium to very strong strength. Anti-LAM1-1, however, did not bind to cells which contained LAM-1 (L + SCR) or LAM-1 (L) alone. The epitope which is recognized by anti-LAM1-1 must, therefore, be composed of a site within the EGF domain, or which contains part of the L and EGF domains, but not the SCR domain. LAM1-3, on the other hand, must only contain the LAM-1(L) domain. The two antibodies are, therefore, distinguishable.

Table 5. Characteristics of Anti-LAM-1 Monoclonal Antibodies

Line	Antibody	TQ1	LAM1-1	LAM1-2	LAM1-3
2	Isotype	G1	G1	M	G1
3	Fluoresc.[1][7]	++	+++	++	++++
4	HEV Bind[2]	N	B	wB	B
5	PPME Bind[3]	B	E	B	B
6	PPME Blocks[4][7]	-	+/-	+/-	-
	Ability to Block Binding of Labelled Monoclonal Antibody[5]				
7	TQ-1	+++	-	+++	+++
8	Leu 8	-	++	-	+++
9	LAM1-1	-	+++	-	-
10	LAM1-3	+++	-	+++	+++
11	Domain Map[6]	L	L+EGF	L	L
	Species Cross Reactivity				
12	Rhesus	-	+++	-	+[1]
13	Tamarin	-	+++	++	--
14	Cow	ND	ND	ND	+++
15	Rabbit	+++	-	++	++++
16	Sheep	-	-	-	+++
17	Dog	-	-	-	+++
18	Cat	-	-	-	+++
19	Pig	ND	ND	ND	+
20	Goat	ND	ND	ND	+++
21	Epitope	C	A	B	D

1 = Fluorescence intensity, human lymphocytes and peripheral mononuclear cells. Fluorescence intensity of staining in indirect immunofluorescence assays is given on a 4-(+) scale where (-) indicates no specific reactivity and (++++) indicates the highest level of activity.

2 = Monoclonal antibody blocking, High Endothelial Venule binding.

3 = Monoclonal antibody blocking, phosphomannan monoester fragments (PPME).

4 = PPME blocking monoclonal antibody.

5 = Ability to block binding of antibody.
6 = Domain mapped.

Abbreviations

Bind = Binding
B = Blocks
wB = weakly Blocks
E = Enhances
ND = Not Done
L = Lectin
EGF = Epidermal Growth Factor-like
SCR = Short Consensus Repeats
N = No effect

Table 6. Structural domains identified by the anti-LAM-1 monoclonal antibody reactive with fusion proteins containing LAM-1 domains[a].

Test mAb	Whole LAM-1	Lectin	Lectin EGF	Lectin EGF SCRs	Lectin SCRs
anti-LAM1-1	+++	-	+++	+++	-
anti-LAM1-2	ND	ND	ND	ND	ND
anti-LAM1-3	+++	+++	++	++++	++

a = Values represent the relative intensity of immunofluorescence staining of COS-7 cells transfected with the LAM-1 cDNA or recombinant cDNAs encoding the lectin, EGF-like, or two SCR domains of LAM-1 with the rest of the cDNAs encoding CD62.
SCR = a domain of short consensus repeats.
EGF = epidermal growth factor-like domain.

We claim:

CLAIMS

1. A hybridoma cell line produced from mouse cells immunized with a mouse pre-B cell line transfected with a human cDNA sequence encoding the leukocyte-adhesion molecule-1, identified as LAM-1, said protein containing domains homologous with binding domains of animal lectin, growth factors and C3/C4 binding proteins.

2. The hybridoma cell line according to claim 1 which produces a monoclonal antibody directed against said LAM-1, said monoclonal antibody blocking (a) lymphocyte traffic to lymph nodes and (b) attachment of leukocytes from blood to endothelium at inflammatory sites.

3. The hybridoma cell line according to claim 1 wherein said immunizing protein is encoded by a LAM-1 cDNA sequence.

4. The hybridoma cell line according to claim 1 wherein said immunizing protein derives from a cDNA sequence that has a nucleotide sequence substantially as indicated in Fig. 2.

5. A hybridoma cell line which produces a monoclonal antibody reactive with a protein derived from a human cDNA sequence encoding the leukocyte-adhesion molecule-1, identified as LAM-1.

6. A monoclonal antibody which reacts with a protein derived from human cDNA sequence encoding the leukocyte-adhesion molecule-1, identified as LAM-1.

7. A monoclonal antibody which binds to the leukocyte-adhesion molecule-1, identified as LAM-1, or immunogenic fragment thereof, said protein or immunogenic fragment containing domains homologous with binding domains of animal lectin, growth factors and C3/C4 binding proteins.

8. The monoclonal antibody according to claim 7 wherein said monoclonal antibody binds to a specific epitope of a protein having the amino acid sequence

substantially as indicated in Fig. 2.

9. The monoclonal antibody according to claim 7 wherein said monoclonal antibody is a chimerized monoclonal antibody having a variable region derived from a mouse monoclonal antibody and constant regions derived from a human antibody.

10. A hybridoma cell line which produces a monoclonal antibody directed against the leukocyte adhesion molecule-1, identified as LAM-1, which monoclonal antibody blocks lymphocyte traffic to lymph nodes and attachment of leukocytes from blood to inflammatory sites.

11. The hybridoma cell line according to claim 10 wherein said cell line produces a monoclonal antibody reactive with the LAM-1 molecule of humans, monkeys, cows, rabbits, sheep, dogs, cats, pigs and goats.

12. The hybridoma cell line according to claim 10 wherein said cell line is produced by the fusion of NS-1 myeloma cells with spleen cells obtained from mice immunized with LAM-1 cDNA transfected cells.

13. A hybridoma cell line having American Type Culture Collection Deposit No. HB 10771.

14. A monoclonal antibody which binds to the leukocyte adhesion molecule-1, identified as LAM-1, said monoclonal antibody blocking lymphocyte traffic to lymph nodes and attachment of leukocytes from blood to inflammatory sites.

15. The monoclonal antibody according to claim 14 wherein said antibody is reactive with LAM-1 of humans, monkeys, cows, rabbits, sheep, dogs, rats, pigs and goats.

16. The monoclonal antibody according to claim 14 wherein said monoclonal antibody reacts with a fusion protein of an LAM-1 domain with a cell protein.

17. The monoclonal antibody according to claim 14 wherein said monoclonal antibody reacts with one selected from the group consisting of (1) a LAM-1 domain and (2) a fusion protein of LAM-1 domains with domains selected

from the group consisting of lectin, epidermal growth factor and short consensus repeats.

18. The monoclonal antibody according to claim 14 wherein said monoclonal antibody reacts with a protein having the LAM-1 domain fused thereon.

19. The monoclonal antibody according to claim 14 wherein said monoclonal antibody is a chimerized monoclonal antibody having a variable region derived from a mouse monoclonal antibody and constant regions derived from a human monoclonal antibody, which chimerized monoclonal antibody binds to the leukocyte adhesion molecule-1, LAM-1; said chimerized monoclonal antibody blocking lyphocyte traffic to lymph nodes and attachment of leukocytes from blood to inflammatory sites.

20. A monoclonal antibody produced by the hybridoma cell line have American Type Culture Collection Deposit No. HB 10771.

21. A hybridoma cell line which produces a monoclonal antibody specific to the antigen identified by the monoclonal antibody produced by the A.T.C.C. hybridoma Deposit No. HB 10771 .

22. A monoclonal antibody specific to the antigen identified by the monoclonal antibody produced by A.T.C.C. hybridoma Deposit No. HB 10771.

23. A chimerized monoclonal antibody specific to the antigen identified by the monoclonal antibody produced by A.T.C.C. Deposit No. HB 10771, said chimerized monoclonal antibody having a variable region derived from a mouse monoclonal antibody which recognizes the antigen identified by the monoclonal antibody produced by A.T.C.C. Deposit No. HB 10771 and constant regions derived from a human monoclonal antibody.

24. A method of separating and/or identifying cells expressing the leukocyte adhesion molecule-1, identified as LAM-1, comprising:

(a) reacting a sample of cells expressing or thought to express LAM-1 with a monoclonal antibody hav-

ing the characteristics of the monoclonal antibody produced by the hybridoma cell line having A.T.C.C. Deposit No. HB 10771; and

(b) separating and/or identifying antibody containing cells from non-antibody containing cells by a separation and/or identification means.

25. A method of separating and/or identifying cells expressing the leukocyte adehesion molecule-1, identified as LAM-1. comprising:

(a) reacting a sample of cells expressing or thought to express LAM-1 with the monoclonal antibody produced by the hybridoma cell line having A.T.C.C. Deposit No. HB 10771 ; and

(b) separating and/or identifying antibody containing cells from non-antibody containing cells by a separation and/or identification means.

26. A method of detecting in an immunoassay the leukocyte adhesion molecule-1, identified as LAM-1, shed by a cell, said method comprising:

(a) contacting a sample containing or thought to contain said LAM-1 molecule with an anti-LAM-1 monoclonal antibody for a time and under conditions sufficient for an antibody/LAM-1 conjugate, if any, to form; and

(b) detecting antibody/LAM-1 conjugate by standard immunoassay methods.

27. The method according to claim 26 wherein said monoclonal antibody is the anti-LAM1-3 monoclonal antibody.

28. The method according to claim 26 wherein said monoclonal antibody is a chimerized monoclonal antibody having a variable region derived from the anti-LAM1-3 monoclonal antibody and constant regions derived from a human antibody.

29. A method of blocking leukocyte mobilization and receptor activity in a patient suffering from a lymphocyte-mobilizing condition, of diminishing leukocyte entry into sites of inflammation, of mitigating or

eliminating autoimmune response, or of overcoming tissue transplant rejection comprising administration of a therapeutic or physiologically effective amount of the anti-LAM1-3 monoclonal antibody to said patient.

30. The method according to claim 29 wherein the monoclonal antibody administered to the patient is a chimerized anti-LAM1-3 monoclonal antibody having a variable region derived from anti-LAM1-3 and constant regions derived from a human antibody.

31. A method of identifying leukocytes with abnormal or decreased expression of LAM-1 for diagnosis and/or detection of leukocyte activation, said method comprising:

(1) incubating an antibody to LAM-1 with a sample containing or thought to contain leukocytes having abnormal or decreased expression of LAM-1; and

(2) identifying leukocytes said abnormal or decreased expression of LAM-1 by standard immunological assay methods selected from the group consisting of flow cytometry, radioimmunoassay and the like.

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Fig. 1A

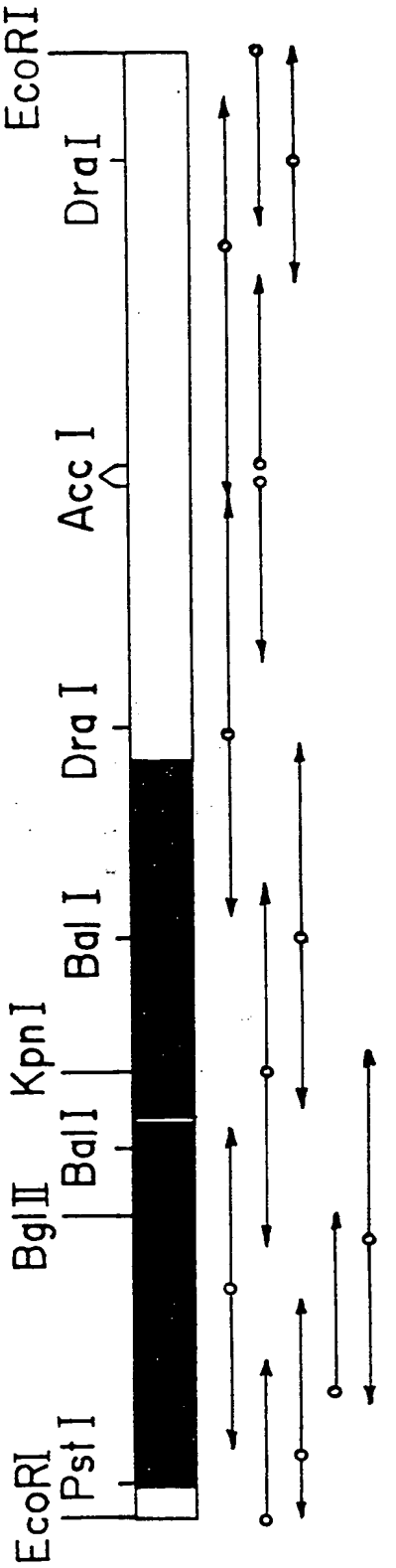
EcoRI
Dra I
Acc I
Dra I
Bal I
Kpn I
Bgl II
Bal I
EcoRI
Pst I

Fig. 1B

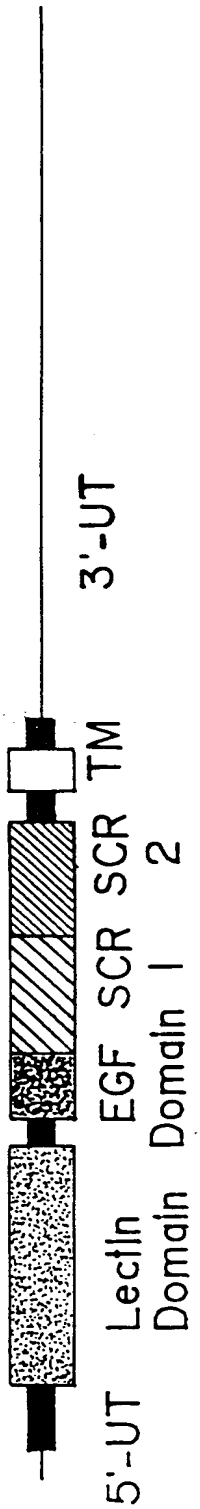
3'-UT
TM
SCR
SCR
EGF
2
Domain I
Domain
Lectin
5'-UT

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Fig. 2

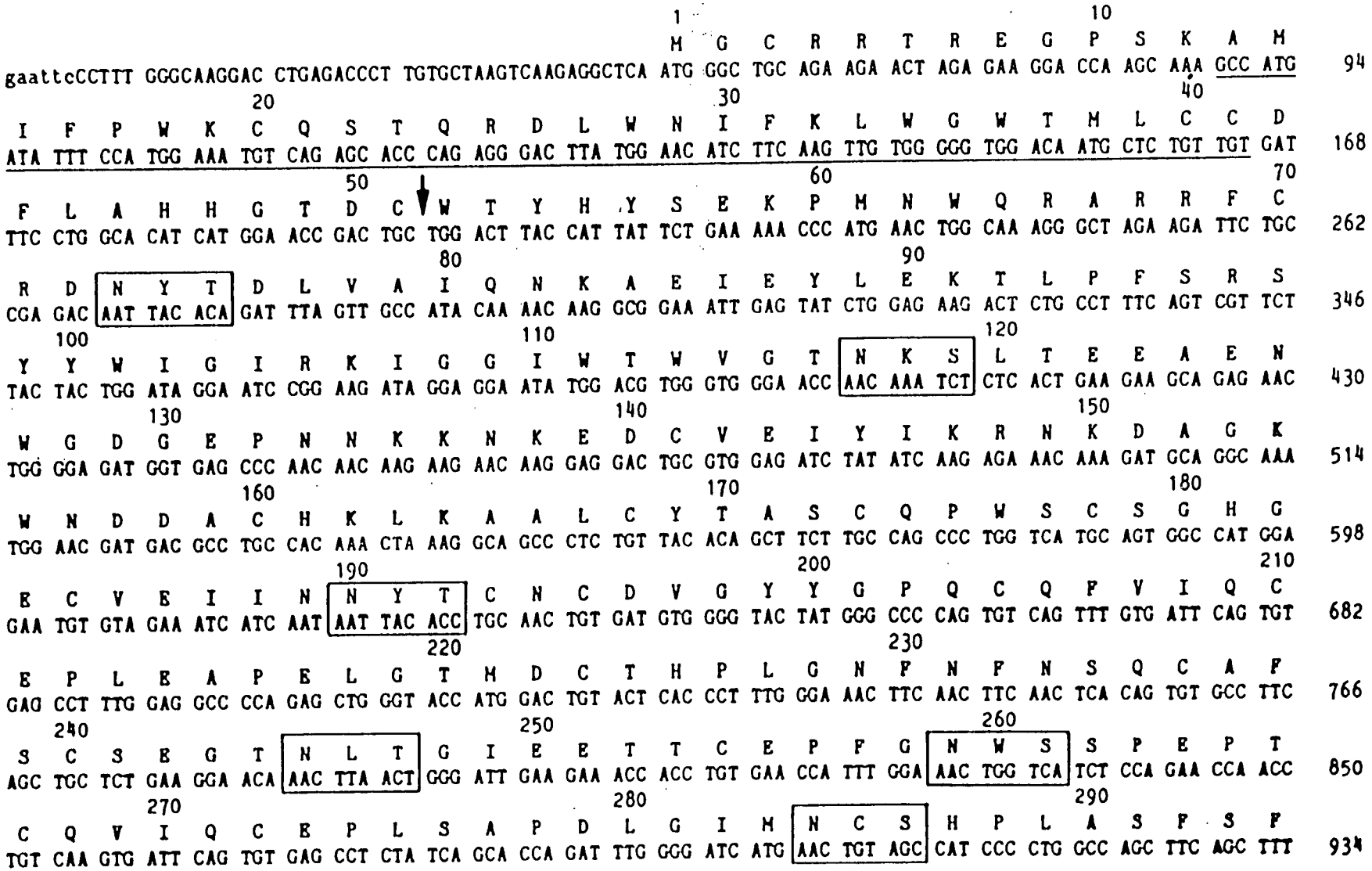

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Fig. 2 (continued)

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                                300                                                                 310                                                                 320
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ACC TCT GCA TGT ACC TTC ATC TGC TCA GAA GGA ACT GAG TTA ATT GGG AAG AAG AAA ACC ATT TGT GAA TCA TCT GGA ATC TGG  1018
                                    330                                                                 340                                                                 350
 S   N   P   S   P   I   C   Q   K   L   D   K   S   F   S   M   I   K   E   G   D   Y   N   P   L   F   I   P
TCA AAT CCT AGT CCA ATA TGT CAA AAA TTG GAC AAA AGT TTC TCA ATG ATT AAG GAG GGT GAT TAT AAC CCC CTC TTC ATT CCA  1102
                                360                                                                 370
 V   A   V   M   V   T   A   F   S   G   L   A   F   I   I   W   L   A   R   R   L   K   K   G   K   K   S   K
GTG GCA GTC ATG GTT ACT GCA TTC TCT GGG TTG GCA TTT ATC ATT TGG CTG GCA AGG AGA TTA AAA AAA GGC AAG AAA TCC AAG  1186
    380
 R   S   M   N   D   P   Y   *
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TCACCCCTCA TTTATCCCTC AACCCCCAGC CCACAGGTGT TTATACAGCT CAGCTTTTTG TCTTTTCTGA GGAGAAACAA ATAAGACCAT AAAGGGAAAG  1480

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AGTGCAAATT TGATACATAT GTGAATATGG ACTCAGTTTT CTTGCAGATC AAATTTCGCG TCGTCTTCTG TATACGTGGA GGTACACTCT ATGAAGTCAA  1680

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GGACCATCCT ATTTAACTGG CTTCAGCCTC CCCACCTTCT TCAGCCACCT CTCTTTTTCA GTTGGCTGAC TTCCACACCT AGCATCTCAT GAGTGCCAAG  1880

CAAAAGGAGA GAAGAGAGAA ATAGCCTGCG CTGTTTTTTA GTTTGGGGGT TTTGCTGTTT CCTTTTATGA GACCCATTCC TATTTCTTAT AGTCAATGTT  1980

TCTTTTATCA CGATATTATT AGTAAGAAAA CATCACTGAA ATGCTAGCTG CAACTGACAT CTCTTTGATG TCATATGGAA GAGTTAAAAC AGGTGGAGAA  2080

ATTCCTTGAT TCACAATGAA ATGCTCTCCT TTCCCCTGCC CCCAGACCTT TTATCCACTT ACCTAGATTC TACATATTCT TTAAATTTCA TCTCAGGCCT  2180

CCCTCAACCC CACCACTTCT TTTATAACTA GTCCTTTACT AATCCAACCC ATGATGAGCT CCTCTTCCTG GCTTCTTACT GAAAGGTTAC CCTGTAACAT  2280

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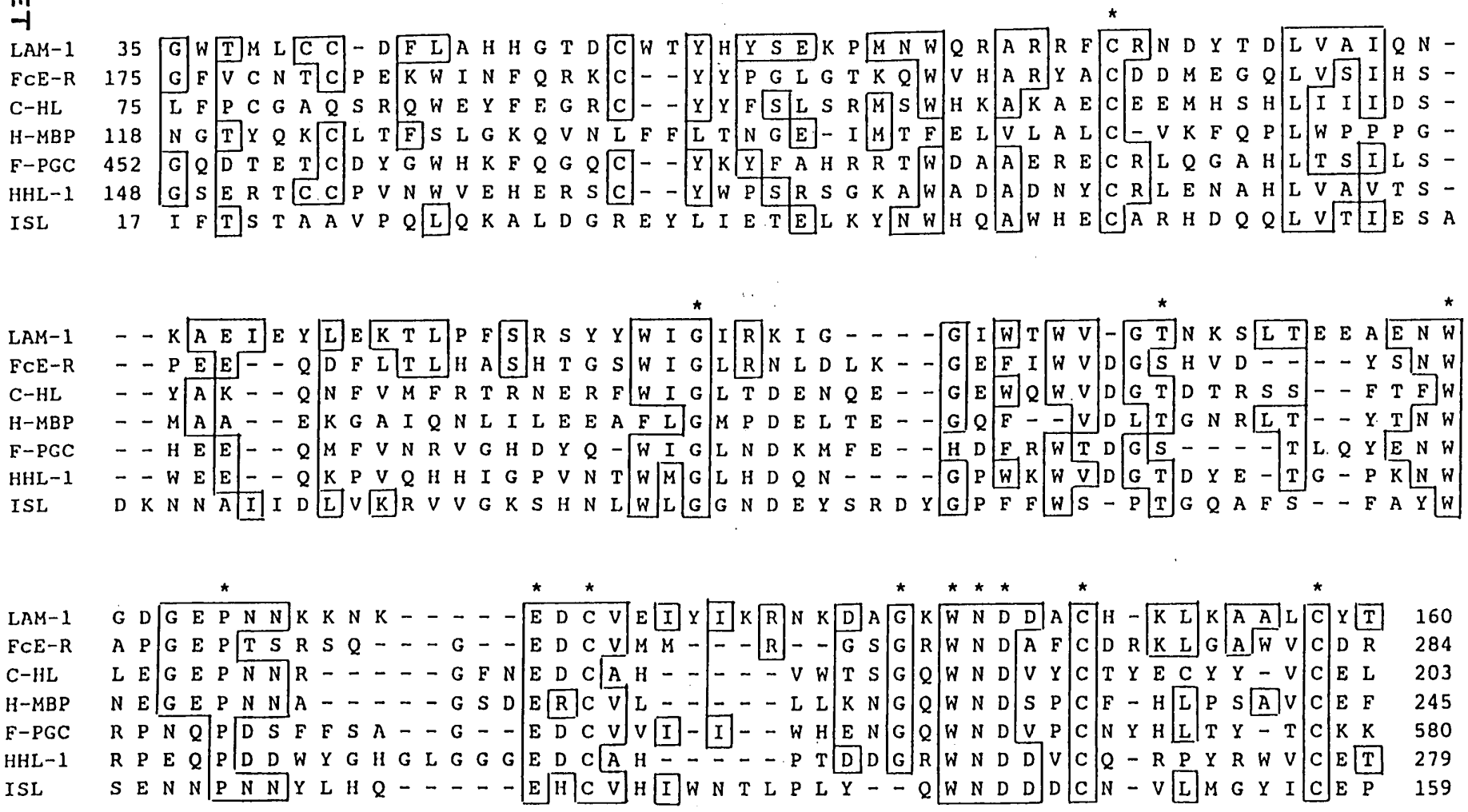
Fig. 3A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FcE-R 175 GFVCNTCPEKWINFQRKC--YYPGLGTKQWVHARYACDDMEGQLVSIHS- 284
C-HL 75 LFPCGAQSRQWEYFEGRC--YYFSLSRMSWHKAKAECEEMHSHLIIIDS- 203
H-MBP 118 NGTYQKCLTFSLGKQVNLFFLTNGE-IMTFELVLALC-VKFQPLWPPPG- 245
F-PGC 452 GQDTETCDYGWHKFQGQC--YKYFAHRRTWDAAERECRLQGAHLTSILS- 580
HHL-1 148 GSERTCCPVNWVEHERSC--YWPSRSGKAWADADNYCRLENAHLVAVTS- 279
ISL 17 IFTSTAAVPQLQKALDGREYLIETELKYNWHQAWHECARHDQQLVTIESA 159
LAM-1 --KAEIEYLEKTLPFSRSYYWIGIRKIG----GIWTWV-GTNKSLTEEAENW
FcE-R --PEE--QDFLTLHASHTGSWIGLRNLDLK--GEFIWVDGSHVD---YSNW
C-HL --YAK--QNFVMFRTRNERFWIGLTDENQE--GEWQWVDGTDTRSS--FTFW
H-MBP --MAA--EKGAIQNLILEEAFLGMPDELTE--GQF--VDLTGNRLT--YTNW
F-PGC --HEE--QMFVNRVGHDYQ-WIGLNDKMFE--HDFRWTDGS----TLQYENW
HHL-1 --WEE--QKPVQHHIGPVNTWMGLHDQN----GPWKWVDGTDYE-TG-PKNW
ISL DKNNAIIDLVKRVVGKSHNLWLGGNDEYSRDYGPFFWS-PTGQAFS--FAYW
LAM-1 GDGEPNNKKNK-----EDCVEIYIKRNKDAGKWNDDAC H-KLKAALCYT
FcE-R APGEPTSRSQ---G--EDCVMM---R--GSGRWNDAFCDRKLGAWVCDR
C-HL LEGEPNNR------GFNEDCAH---VWTSGQWNDVYCTYECYY-VCEL
H-MBP NEGEPNNA------GSDERCVL---LLKNGQWNDSPCF-HLPSAVCEF
F-PGC RPNQPDSFFSA--G--EDCVVI-I--WHENGQWNDVPCNYHLTY-TCKK
HHL-1 RPEQPDDWYGHGLGGGEDCAH------PTDDGRWNDDVCQ-RPYRWVCET
ISL SENNPNNYLHQ-----EHCVHIWNTLPLY--QWNDDDCN-VLMGYICEP

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Fig. 3C

LAM-1 207 V I Q C E P L E A P E L G T M D C T H P L G N F N F N - S Q C A F - - -
LAM-1 269 V I Q C E P L S A P D L G I M N C S H P L A S F S F T - S A C T F - - -
Ba 137 A G Y C S N P G I P - I G T R K V G S Q Y R L E D - - S V - T Y - - -
CR1 694 V - - C Q P P E I L H - G E H T P S H Q D - N F S - P G Q E V F Y - - -
IL-2R 101 P G H C R E P P - P W E N E A T E R I Y H F V V G - Q M V Y Y Q - - -
F-XIII 1 E K P C G F P H V E N G R I A Q Y Y T F K S F Y F P M S I D K K L S F
LAM-1 S C S E G T N - L - T G I E E - T T C - - - E P F G N W S S P E P T C Q 268
LAM-1 I C S E G T E - L - I G K K K - T I C - - - E S S G I W S N P S P I C Q 330
Ba H C S R G L T - L - R G S Q R - R T C - - - Q E G G S W S G T E P S C Q 194
CR1 S C E P G Y D - L - R G A A S - L H C - - - T P Q G D W S D E A P R C A 751
IL-2R - C V Q G Y R A L H R G P A E - S V C K M T H G K T R W T Q P Q L I C T 164
F-XIII F C L A G Y T T E S S R Q E E Q T T C T - T E - - G - W S - P E P R C F 68

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Fig. 4

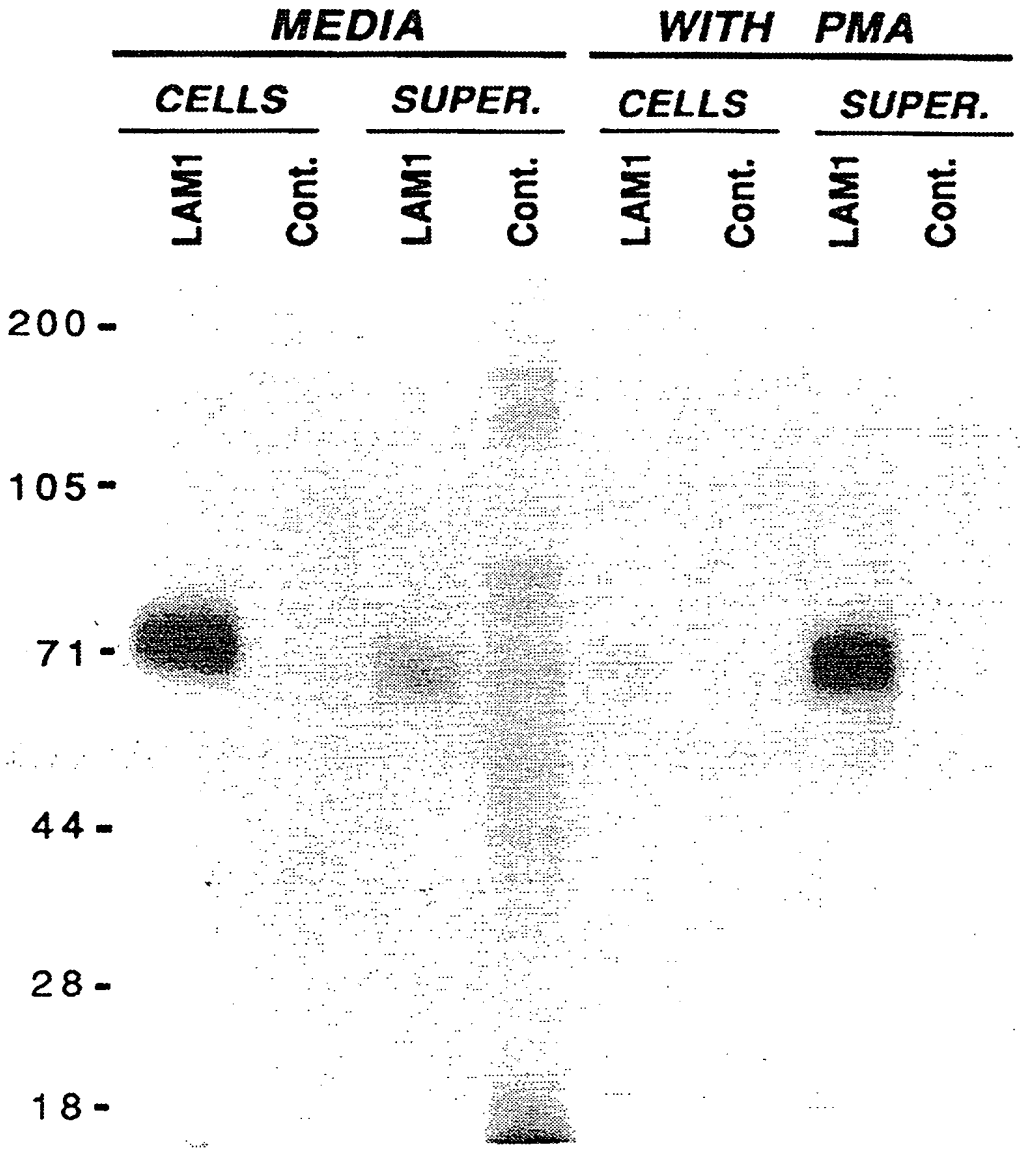
MEDIA
WITH PMA
CELLS
SUPER.
CELLS
SUPER.
LAM1
Cont.
LAM1
Cont.
LAM1
Cont.
LAM1
Cont.
200 -
105 -
71 -
44 -
28 -
18 -

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Fig. 5

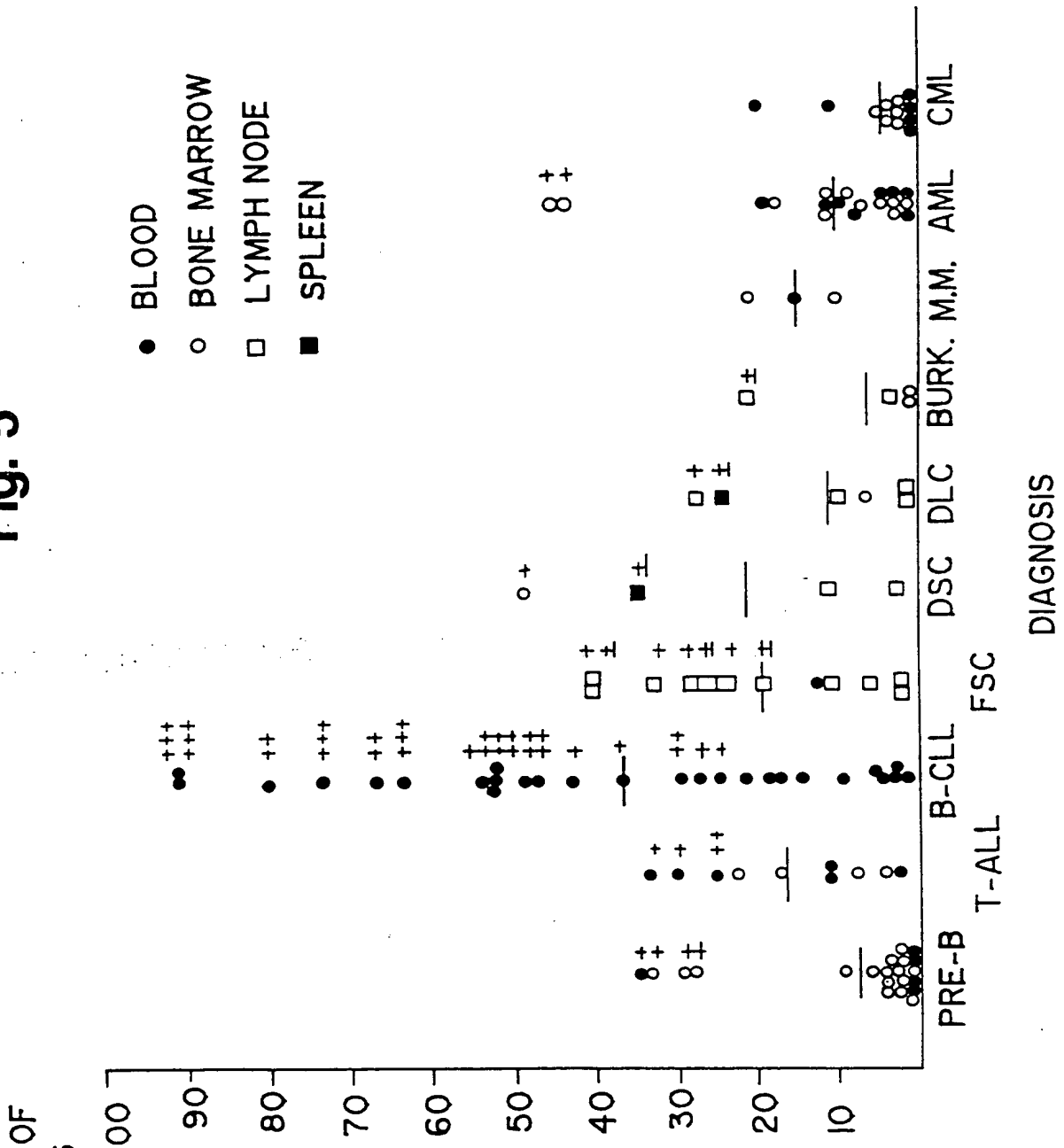
BLOOD
BONE MARROW
LYMPH NODE
SPLEEN
PERCENTAGE OF LAM-1+ CELLS
100
90
80
70
60
50
40
30
20
10
PRE-B
T-ALL
B-CLL
FSC
DSC
DLC
BURK.
M.M.
AML
CML
DIAGNOSIS

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Fig. 6

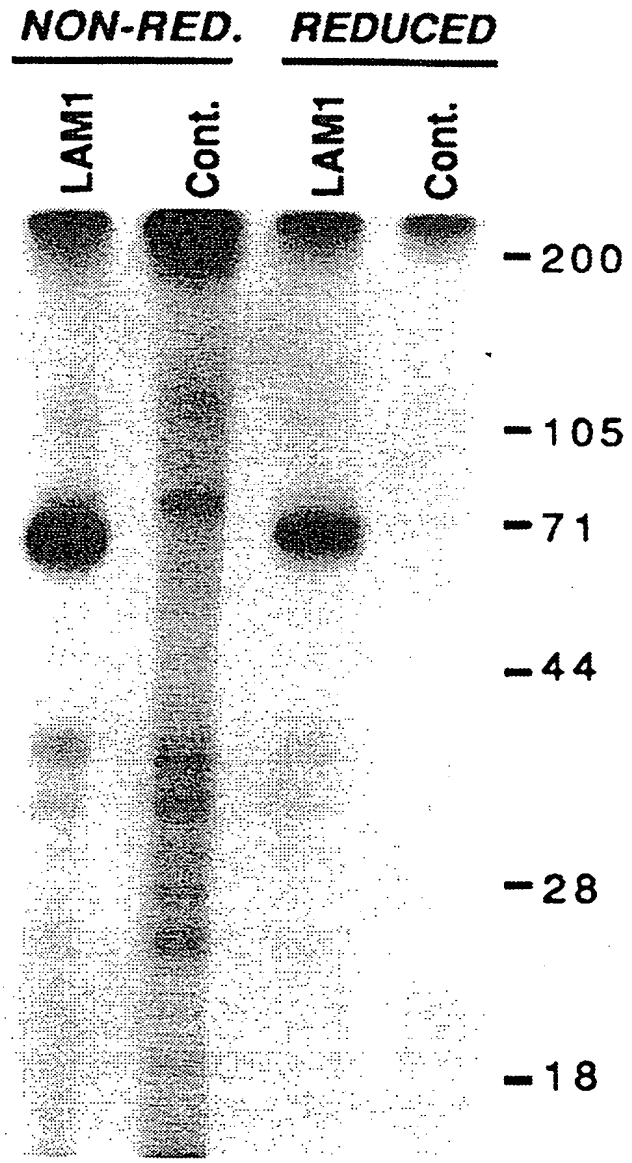

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Fig.7A

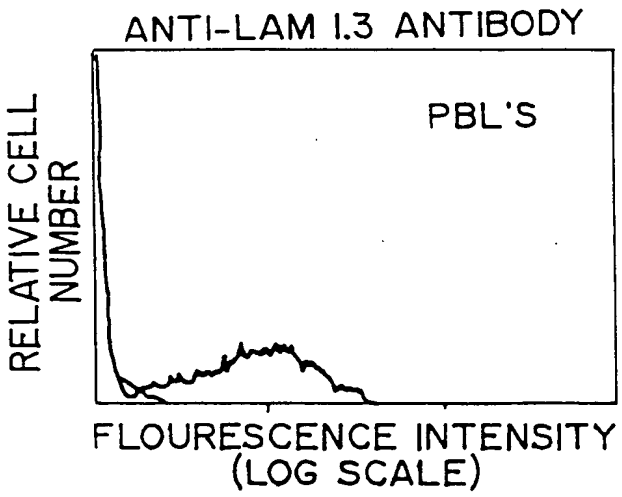

Fig.7B

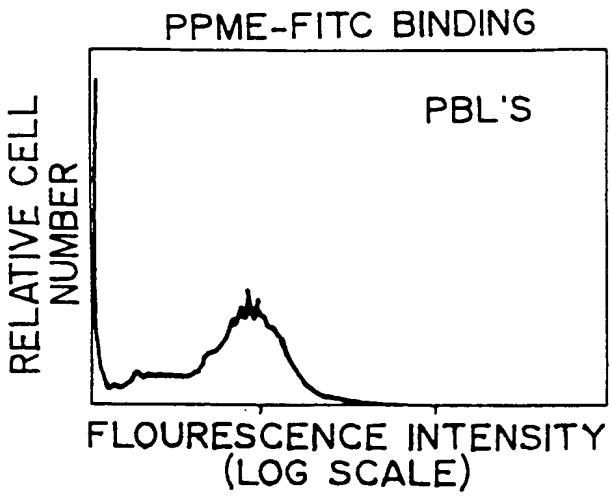

Fig.7C

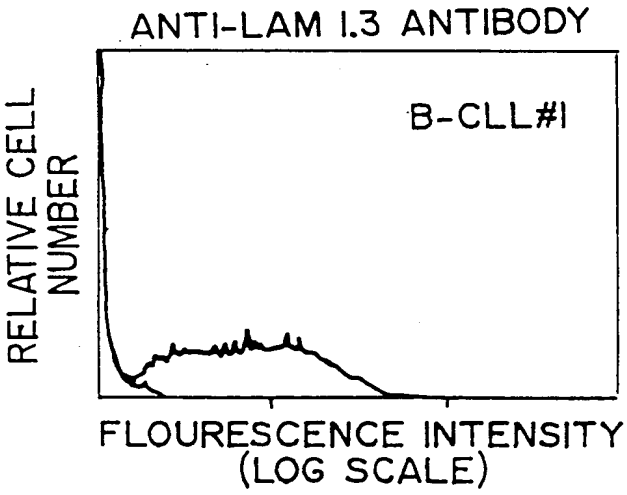

Fig.7D

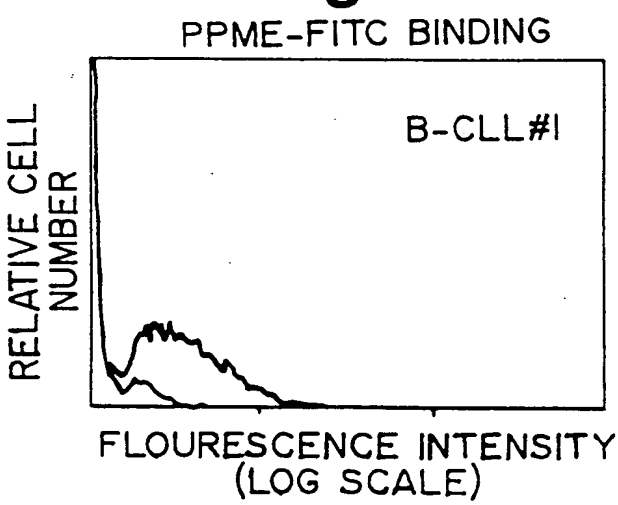

Fig.7E

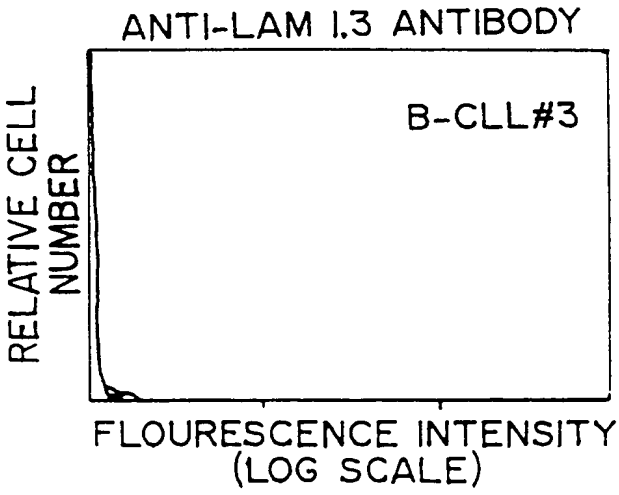

Fig.7F

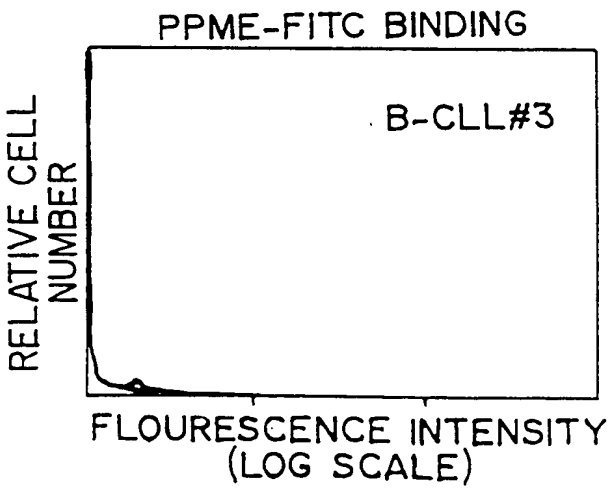

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Fig.8A

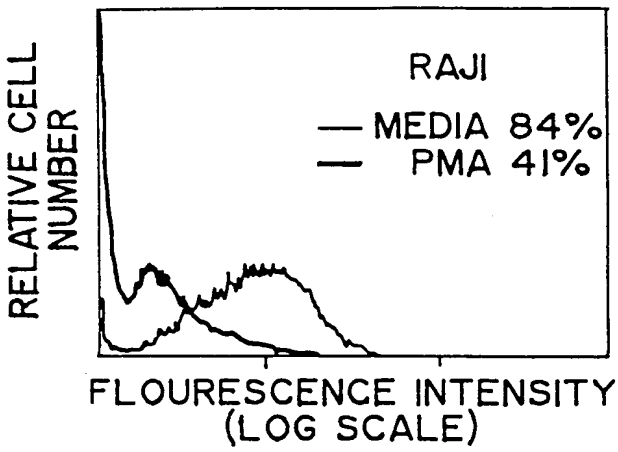


Fig.8B

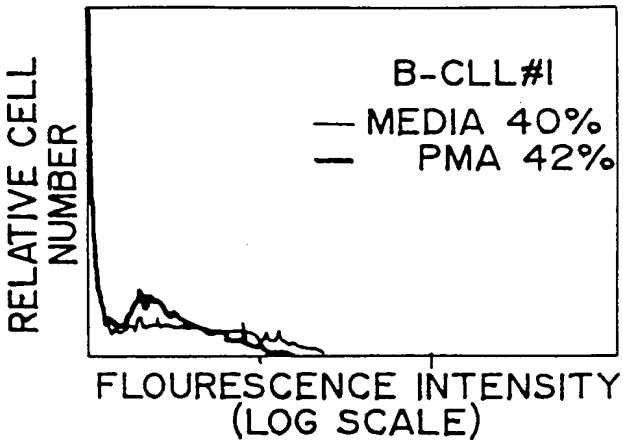


Fig.8C

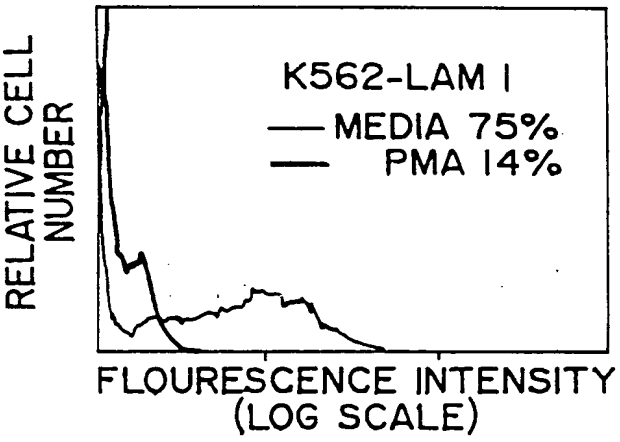


Fig.8D

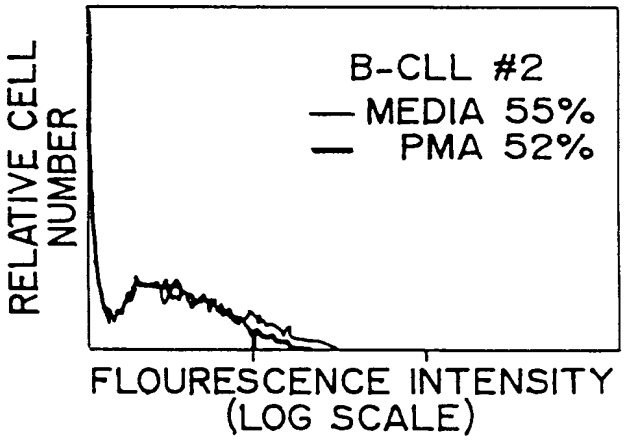


Fig.8E

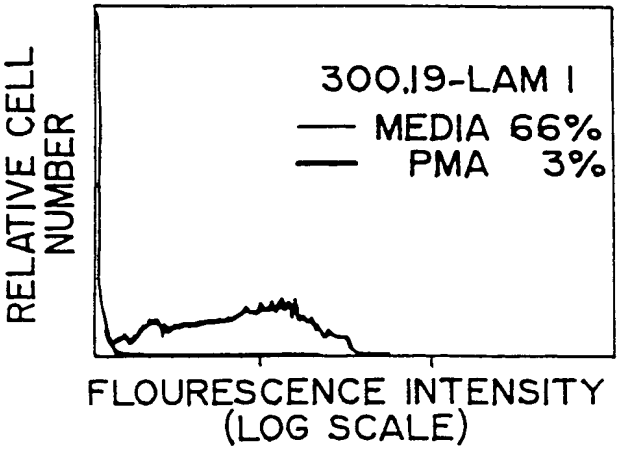
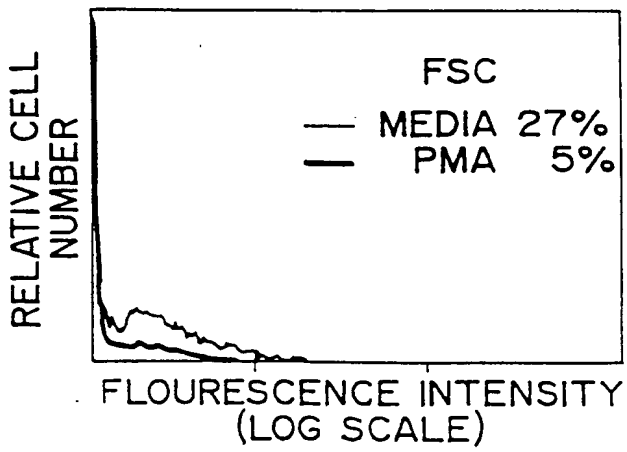


Fig.8F



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[M07K203:00]-[M07K209:00]-

- 2- *- C07K16/28C

SEARCH REPORT

Application Number

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DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.5)
X	LEUKEMIA vol. 5, no. 4 , April 1991 , OXFORD, GB pages 300 - 308 O. SPERTINI ET AL. 'Regulation of leukocyte adhesion molecule-1 (TQ1, Leu-8) expression and shedding by normal and malignant cells.' * abstract * * page 300, right column, line 47 - line 52 * ---	1-8, 10-15, 17,20, 21, 24-27, 31-34	C12N5/20 C12P21/08 C07K3/00 G01N33/577 A61K39/395 G01N33/53
X	EP-A-0 386 906 (DANA-FARBER CANCER INSTITUTE) * the whole document * ---	6,14	
X	THE JOURNAL OF IMMUNOLOGY vol. 144, no. 2 , 15 January 1990 , BALTIMORE MD, USA pages 532 - 540 T. TEDDER ET AL. 'Expression of the human leukocyte adhesion molecule, LAM1.' * abstract * * figure 6 * --- -/--	1-7, 9-12, 14-18, 26,31,32	

TECHNICAL FIELDS SEARCHED (Int.Cl.5)

C12N
C12P
C07K
G01N
A61K

INCOMPLETE SEARCH

The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims

Claims searched completely :

Claims searched incompletely :

Claims not searched :

Reason for the limitation of the search:

see sheet C

1

Place of search	Date of completion of the search	Examiner
THE HAGUE	11 August 1994	Nooij, F

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

Application Number
EP 92 91 5215

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.5)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
P,X	THE FASEB JOURNAL vol. 6, no. 5 , 28 February 1992 , BETHESDA MD, USA page A1889 L. FLAHERTY ET AL. 'A monoclonal antibody (MAb) to L-selectin does not prevent neutrophil (PMN) emigration into an inflamed peritoneal cavity.' * abstract 5514 * ---	6,10,14, 20, 24-27, 29,31,34	
P,X	THE JOURNAL OF CELL BIOLOGY vol. 114, no. 2 , July 1991 , NEW YORK NY, USA pages 351 - 358 G. KANSAS ET AL. 'Molecular mapping of functional domains of the leukocyte receptor for endothelium, LAM-1.' * abstract * * page 352, left column, line 50 - line 56 * * table 1 * -----	1-8,10, 12-14, 16-18, 20,21, 24-27, 31-34	TECHNICAL FIELDS SEARCHED (Int.Cl.5)

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CLAIMS

1. A hybridoma cell line produced from mouse cells immunized with a mouse pre-B cell line transfected with a human cDNA sequence encoding the L-selectin leukocyte-adhesion molecule-1, identified as LAM-1, which contains domains homologous with C-type lectin, epidermal growth factor and consensus repeat units characteristic of complement-binding proteins.

2. The hybridoma cell line according to claim 1 which produces a monoclonal antibody directed against said LAM-1, said monoclonal antibody intended to block (a) lymphocyte traffic to lymph nodes and (b) attachment of leukocytes from blood to endothelium at inflammatory sites.

3. The hybridoma cell line according to claim 1 wherein said immunizing protein is encoded by a LAM-1 cDNA sequence.

4. The hybridoma cell line according to claim 1 wherein said immunizing protein derives from a cDNA sequence that has a nucleotide sequence substantially as indicated in Fig. 2.

5. A hybridoma cell line which produces a monoclonal antibody which specifically binds to a protein derived from a human cDNA sequence encoding the leukocyte-adhesion molecule-1, identified as LAM-1.

6. A monoclonal antibody which specifically binds to a protein derived from human cDNA sequence encoding the leukocyte-adhesion molecule-1, identified as LAM-1.

7. A monoclonal antibody which binds to the L-selectin leukocyte-adhesion molecule-1, identified as LAM-1, or immunogenic fragment thereof, which molecule or immunogenic fragment contains domains homologous with C-type lectin, epidermal growth factor and consensus repeat units characteristic of complement-binding proteins, said monoclonal antibody produced from a hybridoma cell line produced from mouse cells immunized with a mouse pre-B

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cell line transfected with a human cDNA sequence encoding LAM-1.

8. The monoclonal antibody according to claim 7 wherein said monoclonal antibody binds to a specific epitope of a protein having the amino acid sequence substantially as indicated in Fig. 2.

9. The monoclonal antibody according to claim 7 wherein said monoclonal antibody is a chimerized monoclonal antibody having a variable region derived from a mouse monoclonal antibody and constant regions derived from a human antibody.

10. A hybridoma cell line which produces a monoclonal antibody directed against the leukocyte adhesion molecule-1, identified as LAM-1, which monoclonal antibody is intended to block lymphocyte traffic to lymph nodes and attachment of leukocytes from blood to inflammatory sites.

11. The hybridoma cell line according to claim 10 wherein said cell line produces a monoclonal antibody which specifically binds to the LAM-1 molecule of humans, monkeys, cows, rabbits, sheep, dogs, cats, pigs and goats.

12. The hybridoma cell line according to claim 10 wherein said cell line is produced by the fusion of NS-1 myeloma cells with spleen cells obtained from mice immunized with LAM-1 cDNA transfected cells.

13. A hybridoma cell line having American Type Culture Collection Deposit No. HB 10771.

14. A monoclonal antibody which binds to the leukocyte adhesion molecule-1, identified as LAM-1, said monoclonal antibody blocking lymphocyte traffic to lymph nodes and attachment of leukocytes from blood to inflammatory sites.

15. The monoclonal antibody according to claim 14 wherein said antibody specifically binds to LAM-1 of humans, monkeys, cows, rabbits, sheep, dogs, rats, pigs and goats.

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16. The monoclonal antibody according to claim 14 wherein said monoclonal antibody specifically binds to an LAM-1 epitope in a fusion protein resulting from the fusion of an LAM-1 domain with a cell protein.

17. The monoclonal antibody according to claim 14 wherein said monoclonal antibody specifically binds to one of the group consisting of (1) an LAM domain in the LAM-1 molecule and (2) an LAM-1 domain in a fusion protein resulting from the fusion of LAM-1 domains with domains selected from the group consisting of C-type lectin, epidermal growth factor and consensus repeat units characteristic of complement-binding proteins.

18. The monoclonal antibody according to claim 14 wherein said monoclonal antibody specifically binds to a protein having an LAM-1 domain fused thereon.

19. The monoclonal antibody according to claim 14 wherein said monoclonal antibody is a chimerized monoclonal antibody having a variable region derived from a LAM-1 specific mouse monoclonal antibody and constant regions derived from a human monoclonal antibody, which chimerized monoclonal antibody binds to the leukocyte adhesion molecule-1, LAM-1; said chimerized monoclonal antibody blocking lyphocyte traffic to lymph nodes and attachment of leukocytes from blood to inflammatory sites.

20. A monoclonal antibody produced by the hybridoma cell line have American Type Culture Collection Deposit No. HB 10771.

21. A hybridoma cell line which produces a monoclonal antibody which specifically binds to the antigen identified by the monoclonal antibody produced by the A.T.C.C. hybridoma Deposit No. HB 10771 .

22. A monoclonal antibody which specifically binds to the antigen identified by A.T.C.C. hybridoma Deposit No. HB 10771, but not to the same epitope as identified by the monoclonal antibody produced by HB 10771.

23. A chimerized monoclonal antibody which specifically binds to the LAM-1 antigen identified by the monoc-

lonal antibody produced by A.T.C.C. Deposit No. HB 10771, said chimerized monoclonal antibody having a variable region derived from a mouse monoclonal antibody which recognizes the antigen identified by the monoclonal antibody produced by A.T.C.C. Deposit No. HB 10771 and a human constant region.

24. A method of separating and/or identifying cells expressing the leukocyte adhesion molecule-1, identified as LAM-1, comprising:

(a) reacting a sample of cells expressing or thought to express LAM-1 with a monoclonal antibody having the characteristics of the monoclonal antibody produced by the hybridoma cell line having A.T.C.C. Deposit No. HB 10771; and

(b) separating and/or identifying antibody containing cells from non-antibody containing cells by a separation and/or identification means.

25. A method of separating and/or identifying cells expressing the leukocyte adehesion molecule-1, identified as LAM-1. comprising:

(a) reacting a sample of cells expressing or thought to express LAM-1 with the monoclonal antibody produced by the hybridoma cell line having A.T.C.C. Deposit No. HB 10771 ; and

(b) separating and/or identifying antibody containing cells from non-antibody containing cells by a separation and/or identification means.

26. A method of detecting in an immunoassay the leukocyte adhesion molecule-1, identified as LAM-1, shed by a cell, said method comprising:

(a) contacting a sample containing or thought to contain said LAM-1 molecule with an anti-LAM-1 monoclonal antibody for a time and under conditions sufficient for an antibody/LAM-1 conjugate, if any, to form; and

(b) detecting antibody/LAM-1 conjugate by standard immunoassay methods.

27. The method according to claim 26 wherein said

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monoclonal antibody is the anti-LAM1-3 monoclonal antibody.

28. The method according to claim 26 wherein said monoclonal antibody is a chimerized monoclonal antibody having a variable region derived from the anti-LAM1-3 monoclonal antibody and constant regions derived from a human antibody.

29. A method of blocking leukocyte mobilization and receptor activity in a patient suffering from a lymphocyte-mobilizing condition, of diminishing leukocyte entry into sites of inflammation, of mitigating or eliminating autoimmune response, or of overcoming tissue transplant rejection comprising administration of a therapeutic or physiologically effective amount of the anti-LAM1-3 monoclonal antibody to said patient.

30. The method according to claim 29 wherein the monoclonal antibody administered to the patient is a chimerized anti-LAM1-3 monoclonal antibody having a variable region derived from anti-LAM1-3 and constant regions derived from a human antibody.

31. A method of identifying leukocytes with abnormal or decreased expression of LAM-1 for diagnosis and/or detection of leukocyte activation, said method comprising:

(1) incubating an antibody to LAM-1 with a sample containing or thought to contain leukocytes having abnormal or decreased expression of LAM-1; and

(2) identifying leukocytes said abnormal or decreased expression of LAM-1 by standard immunological assay methods selected from the group consisting of flow cytometry, radioimmunoassay and the like.

32. A hybridoma cell line produced from mouse cells immunized with a cell line transfected with a human cDNA sequence encoding the L-selectin leukocyte-adhesion molecule-1, identified as LAM-1, which molecule contains domains homologous with C-type lectin, epidermal growth factor and consensus repeat units characteristic of

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complement-binding protein.

33. A hybridoma cell line which produces a monoclonal antibody specific to the eptitope identified by the monoclonal antibody produced by A.T.C.C. hybridoma Deposit No. HB 10771.

34. A monoclonal antibody specific to the eptitope identified by the monoclonal antibody developed from A.T.C.C. Deposit No. HB 10771.

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Remark : Although claims 29 and 30 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

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